

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
10 May 2001 (10.05.2001)

PCT

(10) International Publication Number  
**WO 01/32707 A1**

(51) International Patent Classification<sup>7</sup>: C07K 14/47,  
C12N 15/12, 15/63, C07K 16/18, C12Q 1/68, C07K  
19/00, G01N 33/53

(21) International Application Number: PCT/US00/30061

(22) International Filing Date:  
1 November 2000 (01.11.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
09/432,333 2 November 1999 (02.11.1999) US

(71) Applicant: ZYMOGENETICS, INC. [US/US]; 1201  
Eastlake Avenue East, Seattle, WA 98102 (US).

(72) Inventor: HOLLOWAY, James, L.; 835 NE 89th St, Seat-  
tle, WA 98115 (US).

(74) Agent: JONES, Phillip, B., C.; ZymoGenetics, Inc., 1201  
Eastlake Avenue East, Seattle, WA 98102 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE,  
DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU,  
ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS,  
LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ,  
PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT,  
TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,  
CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**

- With international search report.
- Before the expiration of the time limit for amending the  
claims and to be republished in the event of receipt of  
amendments.

*For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.*

(54) Title: HUMAN SEMAPHORIN

(57) Abstract: The semaphorin family includes both secreted and membrane proteins. These proteins provide guidance signals to migrating axons during development, and appear to exert effects in the immune system. Zsmf13 is a new member of this group of proteins.

WO 01/32707 A1

## HUMAN SEMAPHORIN

5

### TECHNICAL FIELD

The present invention relates generally to a new polypeptide having  
10 diagnostic and therapeutic uses. In particular, the present invention relates to a novel polypeptide, designated "Zsmf13," and to nucleic acid molecules encoding Zsmf13.

### BACKGROUND OF THE INVENTION

15 Cellular differentiation of multicellular organisms is controlled by hormones and polypeptide growth factors. These diffusable molecules allow cells to communicate with each other and act in concert to form tissues and organs, and to repair and regenerate damaged tissue. Examples of hormones and growth factors include the steroid hormones, parathyroid hormone, follicle stimulating hormone, the  
20 interferons, the interleukins, platelet derived growth factor, epidermal growth factor, and granulocyte-macrophage colony stimulating factor.

Hormones and growth factors influence cellular metabolism by binding to receptor proteins. Certain receptors are integral membrane proteins that bind with the hormone or growth factor outside the cell, and that are linked to signaling pathways  
25 within the cell, such as second messenger systems. Other classes of receptors are soluble intracellular molecules.

Particular examples of biologically active polypeptides include extracellular matrix and cell surface molecules that promote the growth of neurites, or that exert repulsive effects on growth cones. The semaphorin family represents one  
30 group of polypeptides characterized by such axon guidance properties (see, for example, Varela-Echavarria and Guthrie, *Genes & Develop.* 11:545 (1997), and Yu and Kolodkin, *Neuron* 22:11 (1999)). Members of the semaphorin family, which comprises both secreted and membrane proteins, provide guidance signals to migrating axons during development. Semaphorins are biologically active in the immune system as well  
35 (Spriggs, *Curr. Opin. Immunol.* 11:387 (1999)).

In view of the significant roles played by such proteins, a need exists for the identification of new semaphorins, which can provide new tools in detecting and treating alterations in the nervous system and immunological system.

5

## SUMMARY OF THE INVENTION

The present invention provides a novel polypeptide, designated "Zsmf13." The present invention also provides Zsmf13 polypeptides and Zsmf13 fusion proteins, as well as nucleic acid molecules encoding such polypeptides and proteins, and methods for using these nucleic acid molecules and amino acid sequences.

10

## DETAILED DESCRIPTION OF THE INVENTION

### 1. Overview

15

The present invention provides nucleic acid molecules that encodes an N-terminal portion of a new human polypeptide, designated as "Zsmf13," that is a new member of the semaphorin family. An illustrative nucleic acid molecule containing a sequence that encodes the Zsmf13 polypeptide has the nucleotide sequence of SEQ ID NO:1. The encoded polypeptide has the following amino acid sequence:

20 MVLAGPLAVS LLLPSLTLLV SHLSSSQDVS SEPSSEQQLC ALSKHPTVAF  
EDLQPWVS NF TYPGARDFSQ LALDPSGNQL IVGARNYLFR LSLANVSL LQ  
ATEWASSED T RRSCQSKGKT EEECCQNYVRV LIVAGRKVFM CGTNAFSPMC  
TSRQVGNLSR TIEKINGVAR CPYDPRHNST AVISSQGELY AATVIDFSGR  
DPAIYRSLGS GPPRAEF(SEQ ID NO:2). Thus, the *Zsmf13* nucleotide sequence  
25 described herein encodes a polypeptide of 218 amino acids. The putative signal sequence of Zsmf13 polypeptide is located at amino acid residues 1 to 19 of SEQ ID NO:2. The *Zsmf13* gene is expressed by adrenal tumor cells.

25

As described below, the present invention provides isolated polypeptides comprising an amino acid sequence that is at least 70%, at least 80%, or at least 90% identical to amino acid residues 20 to 218 of SEQ ID NO:2, wherein such isolated polypeptides can specifically bind with an antibody that specifically binds with a polypeptide consisting of the amino acid sequence of SEQ ID NO:2. An illustrative polypeptide is a polypeptide that comprises the amino acid sequence of SEQ ID NO:2.

30

The present invention also provides peptides and polypeptides, which  
35 comprise 15, 20, or 30 contiguous amino acid residues of SEQ ID NO:2, amino acid

residues 20 to 218 of SEQ ID NO:2, amino acid residues 20 to 132 of SEQ ID NO:2, or amino acid residues 200 to 218 of SEQ ID NO:2.

The present invention further provides antibodies and antibody fragments that specifically bind with such polypeptides. Exemplary antibodies include polyclonal antibodies, murine monoclonal antibodies, humanized antibodies derived from murine monoclonal antibodies, and human monoclonal antibodies. Illustrative antibody fragments include F(ab')<sub>2</sub>, F(ab)<sub>2</sub>, Fab', Fab, Fv, scFv, and minimal recognition units. The present invention also includes anti-idiotypic antibodies that specifically bind with such antibodies or antibody fragments. The present invention further includes compositions comprising a carrier and a peptide, polypeptide, antibody, or anti-idiotypic antibody described herein.

The present invention also provides isolated nucleic acid molecules that encode a Zsmf13 polypeptide, wherein the nucleic acid molecule is selected from the group consisting of (a) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:3, (b) a nucleic acid molecule encoding the amino acid sequence of SEQ ID NO:2, and (c) a nucleic acid molecule that remains hybridized following stringent wash conditions to a nucleic acid molecule consisting of the nucleotide sequence of nucleotides 168 to 821 SEQ ID NO:1, or the complement of the nucleotide sequence of nucleotides 168 to 821 of SEQ ID NO:1.

Illustrative nucleic acid molecules include those in which any difference between the amino acid sequence encoded by the nucleic acid molecule and the corresponding amino acid sequence of SEQ ID NO:2 is due to a conservative amino acid substitution. The present invention further contemplates isolated nucleic acid molecules that comprise a nucleotide sequence of nucleotides 168 to 821 of SEQ ID NO:1.

The present invention also includes vectors and expression vectors comprising such nucleic acid molecules. Such expression vectors may comprise a transcription promoter, and a transcription terminator, wherein the promoter is operably linked with the nucleic acid molecule, and wherein the nucleic acid molecule is operably linked with the transcription terminator. The present invention further includes recombinant host cells comprising these vectors and expression vectors. Illustrative host cells include bacterial, yeast, avian, fungal, insect, mammalian, and plant cells. Recombinant host cells comprising such expression vectors can be used to prepare Zsmf13 polypeptides by culturing such recombinant host cells that comprise the expression vector and that produce the Zsmf13 protein, and, optionally, isolating the Zsmf13 protein from the cultured recombinant host cells. In addition, the present invention provides pharmaceutical compositions comprising a pharmaceutically

acceptable carrier and at least one of such an expression vector or recombinant virus comprising such expression vectors.

The present invention also contemplates methods for detecting the presence of *Zsmf13* RNA in a biological sample, comprising the steps of (a) contacting  
5 a *Zsmf13* nucleic acid probe under hybridizing conditions with either (i) test RNA molecules isolated from the biological sample, or (ii) nucleic acid molecules synthesized from the isolated RNA molecules, wherein the probe has a nucleotide sequence comprising a portion of the nucleotide sequence of SEQ ID NO:1, or its complement, and (b) detecting the formation of hybrids of the nucleic acid probe and  
10 either the test RNA molecules or the synthesized nucleic acid molecules, wherein the presence of the hybrids indicates the presence of *Zsmf13* RNA in the biological sample. An exemplary biological sample is a human biological sample, such as a biopsy or autopsy specimen.

The present invention further provides methods for detecting the  
15 presence of *Zsmf13* polypeptide in a biological sample, comprising the steps of: (a) contacting the biological sample with an antibody or an antibody fragment that specifically binds with a polypeptide consisting of the amino acid sequence of SEQ ID NO:2, wherein the contacting is performed under conditions that allow the binding of the antibody or antibody fragment to the biological sample, and (b) detecting any of the  
20 bound antibody or bound antibody fragment. Such an antibody or antibody fragment may further comprise a detectable label selected from the group consisting of radioisotope, fluorescent label, chemiluminescent label, enzyme label, bioluminescent label, and colloidal gold. An exemplary biological sample is a human biological sample, such as a biopsy or autopsy specimen.

25 The present invention also provides kits for performing these detection methods. For example, a kit for detection of *Zsmf13* gene expression may comprise a container that comprises a nucleic acid molecule, wherein the nucleic acid molecule is selected from the group consisting of (a) a nucleic acid molecule comprising the nucleotide sequence of nucleotides 168 to 821 of SEQ ID NO:1, (b) a nucleic acid  
30 molecule comprising the complement of nucleotides 168 to 821 of the nucleotide sequence of SEQ ID NO:1, (c) a nucleic acid molecule that is a fragment of (a) consisting of at least eight nucleotides, and (d) a nucleic acid molecule that is a fragment of (b) consisting of at least eight nucleotides. Such a kit may also comprise a second container that comprises one or more reagents capable of indicating the presence  
35 of the nucleic acid molecule. On the other hand, a kit for detection of *Zsmf13* protein may comprise a container that comprises an antibody, or an antibody fragment, that

specifically binds with a polypeptide consisting of the amino acid sequence of SEQ ID NO:2.

The present invention also contemplates anti-idiotypic antibodies, or anti-idiotypic antibody fragments, that specifically bind an antibody or antibody fragment  
5 that specifically binds a polypeptide consisting of the amino acid sequence of SEQ ID NO:2.

The present invention further provides variant Zsmf13 polypeptides, which comprise an amino acid sequence that shares an identity with the amino acid sequence of SEQ ID NO:2 selected from the group consisting of at least 70% identity,  
10 at least 80% identity, at least 90% identity, at least 95% identity, or greater than 95% identity, and wherein any difference between the amino acid sequence of the variant polypeptide and the amino acid sequence of SEQ ID NO:2 is due to one or more conservative amino acid substitutions.

The present invention also provides fusion proteins comprising a  
15 Zsmf13 polypeptide moiety. Such fusion proteins can further comprise an immunoglobulin moiety. In such fusion proteins, the immunoglobulin moiety may be an immunoglobulin heavy chain constant region, such as a human F<sub>C</sub> fragment. The present invention further includes isolated nucleic acid molecules that encode such fusion proteins.

20

These and other aspects of the invention will become evident upon reference to the following detailed description. In addition, various references are identified below.

## 25    **2.    Definitions**

In the description that follows, a number of terms are used extensively. The following definitions are provided to facilitate understanding of the invention.

As used herein, "nucleic acid" or "nucleic acid molecule" refers to  
30 polynucleotides, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), oligonucleotides, fragments generated by the polymerase chain reaction (PCR), and fragments generated by any of ligation, scission, endonuclease action, and exonuclease action. Nucleic acid molecules can be composed of monomers that are naturally-occurring nucleotides (such as DNA and RNA), or analogs of naturally-occurring  
35 nucleotides (*e.g.*,  $\alpha$ -enantiomeric forms of naturally-occurring nucleotides), or a combination of both. Modified nucleotides can have alterations in sugar moieties

and/or in pyrimidine or purine base moieties. Sugar modifications include, for example, replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, and azido groups, or sugars can be functionalized as ethers or esters. Moreover, the entire sugar moiety can be replaced with sterically and electronically similar structures, such as aza-sugars and carbocyclic sugar analogs. Examples of modifications in a base moiety include alkylated purines and pyrimidines; acylated purines or pyrimidines, or other well-known heterocyclic substitutes. Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages. Analogs of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like. The term "nucleic acid molecule" also includes so-called "peptide nucleic acids," which comprise naturally-occurring or modified nucleic acid bases attached to a polyamide backbone. Nucleic acids can be either single stranded or double stranded.

The term "complement of a nucleic acid molecule" refers to a nucleic acid molecule having a complementary nucleotide sequence and reverse orientation as compared to a reference nucleotide sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

The term "contig" denotes a nucleic acid molecule that has a contiguous stretch of identical or complementary sequence to another nucleic acid molecule. Contiguous sequences are said to "overlap" a given stretch of a nucleic acid molecule either in their entirety or along a partial stretch of the nucleic acid molecule.

The term "degenerate nucleotide sequence" denotes a sequence of nucleotides that includes one or more degenerate codons as compared to a reference nucleic acid molecule that encodes a polypeptide. Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (*i.e.*, GAU and GAC triplets each encode Asp).

The term "structural gene" refers to a nucleic acid molecule that is transcribed into messenger RNA (mRNA), which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

An "isolated nucleic acid molecule" is a nucleic acid molecule that is not integrated in the genomic DNA of an organism. For example, a DNA molecule that encodes a growth factor that has been separated from the genomic DNA of a cell is an isolated DNA molecule. Another example of an isolated nucleic acid molecule is a chemically-synthesized nucleic acid molecule that is not integrated in the genome of an organism. A nucleic acid molecule that has been isolated from a particular species is smaller than the complete DNA molecule of a chromosome from that species.

A "nucleic acid molecule construct" is a nucleic acid molecule, either single- or double-stranded, that has been modified through human intervention to contain segments of nucleic acid combined and juxtaposed in an arrangement not existing in nature.

5 "Linear DNA" denotes non-circular DNA molecules having free 5' and 3' ends. Linear DNA can be prepared from closed circular DNA molecules, such as plasmids, by enzymatic digestion or physical disruption.

"Complementary DNA (cDNA)" is a single-stranded DNA molecule that is formed from an mRNA template by the enzyme reverse transcriptase. Typically, a  
10 primer complementary to portions of mRNA is employed for the initiation of reverse transcription. Those skilled in the art also use the term "cDNA" to refer to a double-stranded DNA molecule consisting of such a single-stranded DNA molecule and its complementary DNA strand. The term "cDNA" also refers to a clone of a cDNA molecule synthesized from an RNA template.

15 A "promoter" is a nucleotide sequence that directs the transcription of a structural gene. Typically, a promoter is located in the 5' non-coding region of a gene, proximal to the transcriptional start site of a structural gene. Sequence elements within promoters that function in the initiation of transcription are often characterized by consensus nucleotide sequences. These promoter elements include RNA polymerase  
20 binding sites, TATA sequences, CAAT sequences, differentiation-specific elements (DSEs; McGehee *et al.*, *Mol. Endocrinol.* 7:551 (1993)), cyclic AMP response elements (CREs), serum response elements (SREs; Treisman, *Seminars in Cancer Biol.* 1:47 (1990)), glucocorticoid response elements (GREs), and binding sites for other transcription factors, such as CRE/ATF (O'Reilly *et al.*, *J. Biol. Chem.* 267:19938  
25 (1992)), AP2 (Ye *et al.*, *J. Biol. Chem.* 269:25728 (1994)), SP1, cAMP response element binding protein (CREB; Loeken, *Gene Expr.* 3:253 (1993)) and octamer factors (see, in general, Watson *et al.*, eds., *Molecular Biology of the Gene*, 4th ed. (The Benjamin/Cummings Publishing Company, Inc. 1987), and Lemaigre and Rousseau, *Biochem. J.* 303:1 (1994)). If a promoter is an inducible promoter, then the rate of  
30 transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter. Repressible promoters are also known.

A "core promoter" contains essential nucleotide sequences for promoter function, including the TATA box and start of transcription. By this definition, a core  
35 promoter may or may not have detectable activity in the absence of specific sequences that may enhance the activity or confer tissue specific activity.



A "regulatory element" is a nucleotide sequence that modulates the activity of a core promoter. For example, a regulatory element may contain a nucleotide sequence that binds with cellular factors enabling transcription exclusively or preferentially in particular cells, tissues, or organelles. These types of regulatory elements are normally associated with genes that are expressed in a "cell-specific," "tissue-specific," or "organelle-specific" manner.

An "enhancer" is a type of regulatory element that can increase the efficiency of transcription, regardless of the distance or orientation of the enhancer relative to the start site of transcription.

"Heterologous DNA" refers to a DNA molecule, or a population of DNA molecules, that does not exist naturally within a given host cell. DNA molecules heterologous to a particular host cell may contain DNA derived from the host cell species (*i.e.*, endogenous DNA) so long as that host DNA is combined with non-host DNA (*i.e.*, exogenous DNA). For example, a DNA molecule containing a non-host DNA segment encoding a polypeptide operably linked to a host DNA segment comprising a transcription promoter is considered to be a heterologous DNA molecule. Conversely, a heterologous DNA molecule can comprise an endogenous gene operably linked with an exogenous promoter. As another illustration, a DNA molecule comprising a gene derived from a wild-type cell is considered to be heterologous DNA if that DNA molecule is introduced into a mutant cell that lacks the wild-type gene.

A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides."

A "protein" is a macromolecule comprising one or more polypeptide chains. A protein may also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is produced, and will vary with the type of cell. Proteins are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

A peptide or polypeptide encoded by a non-host DNA molecule is a "heterologous" peptide or polypeptide.

An "integrated genetic element" is a segment of DNA that has been incorporated into a chromosome of a host cell after that element is introduced into the cell through human manipulation. Within the present invention, integrated genetic elements are most commonly derived from linearized plasmids that are introduced into

the cells by electroporation or other techniques. Integrated genetic elements are passed from the original host cell to its progeny.

A "cloning vector" is a nucleic acid molecule, such as a plasmid, cosmid, or bacteriophage, that has the capability of replicating autonomously in a host cell. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites that allow insertion of a nucleic acid molecule in a determinable fashion without loss of an essential biological function of the vector, as well as nucleotide sequences encoding a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

An "expression vector" is a nucleic acid molecule encoding a gene that is expressed in a host cell. Typically, an expression vector comprises a transcription promoter, a gene, and a transcription terminator. Gene expression is usually placed under the control of a promoter, and such a gene is said to be "operably linked to" the promoter. Similarly, a regulatory element and a core promoter are operably linked if the regulatory element modulates the activity of the core promoter.

A "recombinant host" is a cell that contains a heterologous nucleic acid molecule, such as a cloning vector or expression vector. In the present context, an example of a recombinant host is a cell that produces Zsmf13 from an expression vector. In contrast, Zsmf13 can be produced by a cell that is a "natural source" of Zsmf13, and that lacks an expression vector.

"Integrative transformants" are recombinant host cells, in which heterologous DNA has become integrated into the genomic DNA of the cells.

A "fusion protein" is a hybrid protein expressed by a nucleic acid molecule comprising nucleotide sequences of at least two genes. For example, a fusion protein can comprise at least part of a Zsmf13 polypeptide fused with a polypeptide that binds an affinity matrix. Such a fusion protein provides a means to isolate large quantities of Zsmf13 using affinity chromatography.

The term "receptor" denotes a cell-associated protein that binds to a bioactive molecule termed a "ligand." This interaction mediates the effect of the ligand on the cell. Receptors can be membrane bound, cytosolic or nuclear; monomeric (*e.g.*, thyroid stimulating hormone receptor, beta-adrenergic receptor) or multimeric (*e.g.*, PDGF receptor, growth hormone receptor, IL-3 receptor, GM-CSF receptor, G-CSF receptor, erythropoietin receptor and IL-6 receptor). Membrane-bound receptors are characterized by a multi-domain structure comprising an extracellular ligand-binding domain and an intracellular effector domain that is typically involved in signal transduction. In certain membrane-bound receptors, the extracellular ligand-binding

domain and the intracellular effector domain are located in separate polypeptides that comprise the complete functional receptor.

In general, the binding of ligand to receptor results in a conformational change in the receptor that causes an interaction between the effector domain and other molecule(s) in the cell, which in turn leads to an alteration in the metabolism of the cell. Metabolic events that are often linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids.

The term "secretory signal sequence" denotes a nucleotide sequence that encodes a peptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

An "isolated polypeptide" is a polypeptide that is essentially free from contaminating cellular components, such as carbohydrate, lipid, or other proteinaceous impurities associated with the polypeptide in nature. Typically, a preparation of isolated polypeptide contains the polypeptide in a highly purified form, *i.e.*, at least about 80% pure, at least about 90% pure, at least about 95% pure, greater than 95% pure, or greater than 99% pure. One way to show that a particular protein preparation contains an isolated polypeptide is by the appearance of a single band following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the protein preparation and Coomassie Brilliant Blue staining of the gel. However, the term "isolated" does not exclude the presence of the same polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

The terms "amino-terminal" and "carboxyl-terminal" are used herein to denote positions within polypeptides. Where the context allows, these terms are used with reference to a particular sequence or portion of a polypeptide to denote proximity or relative position. For example, a certain sequence positioned carboxyl-terminal to a reference sequence within a polypeptide is located proximal to the carboxyl terminus of the reference sequence, but is not necessarily at the carboxyl terminus of the complete polypeptide.

The term "expression" refers to the biosynthesis of a gene product. For example, in the case of a structural gene, expression involves transcription of the structural gene into mRNA and the translation of mRNA into one or more polypeptides.

The term "splice variant" is used herein to denote alternative forms of RNA transcribed from a gene. Splice variation arises naturally through use of

complement/anti-complement pair. Other exemplary complement/anti-complement  
10 pairs include receptor/ligand pairs, antibody/antigen (or hapten or epitope) pairs,  
sense/antisense polynucleotide pairs, and the like. Where subsequent dissociation of  
the complement/anti-complement pair is desirable, the complement/anti-complement  
pair preferably has a binding affinity of less than  $10^9 \text{ M}^{-1}$ .

An "anti-idiotypic antibody" is an antibody that binds with the variable  
15 region domain of an immunoglobulin. In the present context, an anti-idiotypic antibody  
binds with the variable region of an anti-Zsmf13 antibody, and thus, an anti-idiotypic  
antibody mimics an epitope of Zsmf13.

An "antibody fragment" is a portion of an antibody such as  $\text{F(ab')}_2$ ,  $\text{F(ab)}_2$ ,  
Fab', Fab, and the like. Regardless of structure, an antibody fragment binds with the same  
20 antigen that is recognized by the intact antibody. For example, an anti-Zsmf13  
monoclonal antibody fragment binds with an epitope of Zsmf13.

The term "antibody fragment" also includes a synthetic or a genetically  
engineered polypeptide that binds to a specific antigen, such as polypeptides consisting of  
the light chain variable region, "Fv" fragments consisting of the variable regions of the  
25 heavy and light chains, recombinant single chain polypeptide molecules in which light  
and heavy variable regions are connected by a peptide linker ("scFv proteins"), and  
minimal recognition units consisting of the amino acid residues that mimic the  
hypervariable region.

A "chimeric antibody" is a recombinant protein that contains the variable  
30 domains and complementary determining regions derived from a rodent antibody, while  
the remainder of the antibody molecule is derived from a human antibody.

"Humanized antibodies" are recombinant proteins in which murine  
complementarity determining regions of a monoclonal antibody have been transferred  
from heavy and light variable chains of the murine immunoglobulin into a human variable  
35 domain.

A "detectable label" is a molecule or atom, which can be conjugated to  
an antibody moiety to produce a molecule useful for diagnosis. Examples of detectable

labels include chelators, photoactive agents, radioisotopes, fluorescent agents, paramagnetic ions, or other marker moieties.

The term "affinity tag" is used herein to denote a polypeptide segment that can be attached to a second polypeptide to provide for purification or detection of the second polypeptide or provide sites for attachment of the second polypeptide to a substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-histidine tract, protein A (Nilsson *et al.*, *EMBO J.* 4:1075 (1985); Nilsson *et al.*, *Methods Enzymol.* 198:3 (1991)), glutathione S transferase (Smith and Johnson, *Gene* 67:31 (1988)), Glu-Glu affinity tag (Grussenmeyer *et al.*, *Proc. Natl. Acad. Sci. USA* 82:7952 (1985)), substance P, FLAG peptide (Hopp *et al.*, *Biotechnology* 6:1204 (1988)), streptavidin binding peptide, or other antigenic epitope or binding domain. See, in general, Ford *et al.*, *Protein Expression and Purification* 2:95 (1991). Nucleic acid molecules encoding affinity tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ).

A "naked antibody" is an entire antibody, as opposed to an antibody fragment, which is not conjugated with a therapeutic agent. Naked antibodies include both polyclonal and monoclonal antibodies, as well as certain recombinant antibodies, such as chimeric and humanized antibodies.

As used herein, the term "antibody component" includes both an entire antibody and an antibody fragment.

A "target polypeptide" or a "target peptide" is an amino acid sequence that comprises at least one epitope, and that is expressed on a target cell, such as a tumor cell, or a cell that carries an infectious agent antigen. T cells recognize peptide epitopes presented by a major histocompatibility complex molecule to a target polypeptide or target peptide and typically lyse the target cell or recruit other immune cells to the site of the target cell, thereby killing the target cell.

An "antigenic peptide" is a peptide, which will bind a major histocompatibility complex molecule to form an MHC-peptide complex which is recognized by a T cell, thereby inducing a cytotoxic lymphocyte response upon presentation to the T cell. Thus, antigenic peptides are capable of binding to an appropriate major histocompatibility complex molecule and inducing a cytotoxic T cells response, such as cell lysis or specific cytokine release against the target cell, which binds or expresses the antigen. The antigenic peptide can be bound in the context of a class I or class II major histocompatibility complex molecule, on an antigen presenting cell or on a target cell.

A “ribozyme” is a nucleic acid molecule that contains a catalytic center. The term includes RNA enzymes, self-splicing RNAs, self-cleaving RNAs, and nucleic acid molecules that perform these catalytic functions. A nucleic acid molecule that  
15 encodes a ribozyme is termed a “ribozyme gene.”

An “external guide sequence” is a nucleic acid molecule that directs the endogenous ribozyme, RNase P, to a particular species of intracellular mRNA, resulting in the cleavage of the mRNA by RNase P. A nucleic acid molecule that encodes an external guide sequence is termed an “external guide sequence gene.”

20 The term “variant *Zsmf13* gene” refers to nucleic acid molecules that encode a polypeptide having an amino acid sequence that is a modification of SEQ ID NO:2. Such variants include naturally-occurring polymorphisms of *Zsmf13* genes, as well as synthetic genes that contain conservative amino acid substitutions of the amino acid sequence of SEQ ID NO:2. Additional variant forms of *Zsmf13* genes are nucleic  
25 acid molecules that contain insertions or deletions of the nucleotide sequences described herein. A variant *Zsmf13* gene can be identified by determining whether the gene hybridizes with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, or its complement, under stringent conditions.

Alternatively, variant *Zsmf13* genes can be identified by sequence  
30 comparison. Two amino acid sequences have “100% amino acid sequence identity” if the amino acid residues of the two amino acid sequences are the same when aligned for maximal correspondence. Similarly, two nucleotide sequences have “100% nucleotide sequence identity” if the nucleotide residues of the two nucleotide sequences are the same when aligned for maximal correspondence. Sequence comparisons can be  
35 performed using standard software programs such as those included in the LASERGENE bioinformatics computing suite, which is produced by DNASTAR (Madison, Wisconsin). Other methods for comparing two nucleotide or amino acid

sequences by determining optimal alignment are well-known to those of skill in the art (see, for example, Peruski and Peruski, *The Internet and the New Biology: Tools for Genomic and Molecular Research* (ASM Press, Inc. 1997), Wu et al. (eds.), "Information Superhighway and Computer Databases of Nucleic Acids and Proteins," 5 in *Methods in Gene Biotechnology*, pages 123-151 (CRC Press, Inc. 1997), and Bishop (ed.), *Guide to Human Genome Computing*, 2nd Edition (Academic Press, Inc. 1998)). Particular methods for determining sequence identity are described below.

Regardless of the particular method used to identify a variant *Zsmf13* gene or variant *Zsmf13* polypeptide, a variant gene or polypeptide encoded by a variant 10 gene may be characterized by its ability to bind specifically to an anti-*Zsmf13* antibody.

The term "allelic variant" is used herein to denote any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or 15 may encode polypeptides having altered amino acid sequence. The term allelic variant is also used herein to denote a protein encoded by an allelic variant of a gene.

The term "ortholog" denotes a polypeptide or protein obtained from one species that is the functional counterpart of a polypeptide or protein from a different species. Sequence differences among orthologs are the result of speciation.

20 "Paralogs" are distinct but structurally related proteins made by an organism. Paralogs are believed to arise through gene duplication. For example,  $\alpha$ -globin,  $\beta$ -globin, and myoglobin are paralogs of each other.

The present invention includes functional fragments of *Zsmf13* genes. Within the context of this invention, a "functional fragment" of a *Zsmf13* gene refers to 25 a nucleic acid molecule that encodes a portion of a *Zsmf13* polypeptide specifically binds with an anti-*Zsmf13* antibody.

Due to the imprecision of standard analytical methods, molecular weights and lengths of polymers are understood to be approximate values. When such a value is expressed as "about" X or "approximately" X, the stated value of X will be 30 understood to be accurate to  $\pm 10\%$ .

### 3. ***Production of Nucleic Acid Molecules Encoding Human Zsmf13***

Nucleic acid molecules encoding a human *Zsmf13* gene can be obtained by screening a human cDNA or genomic library using polynucleotide probes based 35 upon SEQ ID NO:1. These techniques are standard and well-established.

5 methods well-known to those of skill in the art. In general, RNA isolation techniques must provide a method for breaking cells, a means of inhibiting RNase-directed degradation of RNA, and a method of separating RNA from DNA, protein, and polysaccharide contaminants. For example, total RNA can be isolated by freezing tissue in liquid nitrogen, grinding the frozen tissue with a mortar and pestle to lyse the cells, extracting the ground tissue with a solution of phenol/chloroform to remove proteins, and  
10 separating RNA from the remaining impurities by selective precipitation with lithium chloride (see, for example, Ausubel *et al.* (eds.), *Short Protocols in Molecular Biology*, 3<sup>rd</sup> Edition, pages 4-1 to 4-6 (John Wiley & Sons 1995) ["Ausubel (1995)"]; Wu *et al.*, *Methods in Gene Biotechnology*, pages 33-41 (CRC Press, Inc. 1997) ["Wu (1997)"]). Alternatively, total RNA can be isolated from tissue by extracting ground tissue with  
15 guanidinium isothiocyanate, extracting with organic solvents, and separating RNA from contaminants using differential centrifugation (see, for example, Chirgwin *et al.*, *Biochemistry* 18:52 (1979); Ausubel (1995) at pages 4-1 to 4-6; Wu (1997) at pages 33-41).

In order to construct a cDNA library, poly(A)<sup>+</sup> RNA must be isolated from  
20 a total RNA preparation. Poly(A)<sup>+</sup> RNA can be isolated from total RNA using the standard technique of oligo(dT)-cellulose chromatography (see, for example, Aviv and Leder, *Proc. Nat'l Acad. Sci. USA* 69:1408 (1972); Ausubel (1995) at pages 4-11 to 4-12).

Double-stranded cDNA molecules are synthesized from poly(A)<sup>+</sup> RNA  
25 using techniques well-known to those in the art. (see, for example, Wu (1997) at pages 41-46). Moreover, commercially available kits can be used to synthesize double-stranded cDNA molecules. For example, such kits are available from Life Technologies, Inc. (Gaithersburg, MD), CLONTECH Laboratories, Inc. (Palo Alto, CA), Promega Corporation (Madison, WI) and STRATAGENE (La Jolla, CA).

30 Various cloning vectors are appropriate for the construction of a cDNA library. For example, a cDNA library can be prepared in a vector derived from bacteriophage, such as a  $\lambda$ gt10 vector. See, for example, Huynh *et al.*, "Constructing and Screening cDNA Libraries in  $\lambda$ gt10 and  $\lambda$ gt11," in *DNA Cloning: A Practical Approach Vol. I*, Glover (ed.), page 49 (IRL Press, 1985); Wu (1997) at pages 47-52.

35 Alternatively, double-stranded cDNA molecules can be inserted into a plasmid vector, such as a PBLUESCRIPT vector (STRATAGENE; La Jolla, CA), a LAMDAGEM-4 (Promega Corp.) or other commercially available vectors. Suitable



cloning vectors also can be obtained from the American Type Culture Collection (Manassas, VA).

To amplify the cloned cDNA molecules, the cDNA library is inserted into a prokaryotic host, using standard techniques. For example, a cDNA library can be introduced into competent *E. coli* DH5 cells, which can be obtained, for example, from Life Technologies, Inc. (Gaithersburg, MD).

A human genomic library can be prepared by means well-known in the art (see, for example, Ausubel (1995) at pages 5-1 to 5-6; Wu (1997) at pages 307-327). Genomic DNA can be isolated by lysing tissue with the detergent Sarkosyl, digesting the lysate with proteinase K, clearing insoluble debris from the lysate by centrifugation, precipitating nucleic acid from the lysate using isopropanol, and purifying resuspended DNA on a cesium chloride density gradient.

DNA fragments that are suitable for the production of a genomic library can be obtained by the random shearing of genomic DNA or by the partial digestion of genomic DNA with restriction endonucleases. Genomic DNA fragments can be inserted into a vector, such as a bacteriophage or cosmid vector, in accordance with conventional techniques, such as the use of restriction enzyme digestion to provide appropriate termini, the use of alkaline phosphatase treatment to avoid undesirable joining of DNA molecules, and ligation with appropriate ligases. Techniques for such manipulation are well-known in the art (see, for example, Ausubel (1995) at pages 5-1 to 5-6; Wu (1997) at pages 307-327).

Nucleic acid molecules that encode a human *Zsmf13* gene can also be obtained using the polymerase chain reaction (PCR) with oligonucleotide primers having nucleotide sequences that are based upon the nucleotide sequences of the human *Zsmf13* gene, as described herein. General methods for screening libraries with PCR are provided by, for example, Yu *et al.*, "Use of the Polymerase Chain Reaction to Screen Phage Libraries," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 211-215 (Humana Press, Inc. 1993). Moreover, techniques for using PCR to isolate related genes are described by, for example, Preston, "Use of Degenerate Oligonucleotide Primers and the Polymerase Chain Reaction to Clone Gene Family Members," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 317-337 (Humana Press, Inc. 1993).

Alternatively, human genomic libraries can be obtained from commercial sources such as Research Genetics (Huntsville, AL) and the American Type Culture Collection (Manassas, VA).

A library containing cDNA or genomic clones can be screened with one or more polynucleotide probes based upon SEQ ID NO:1, using standard methods (see, for example, Ausubel (1995) at pages 6-1 to 6-11).

Anti-Zsmf13 antibodies, produced as described below, can also be used to isolate DNA sequences that encode human *Zsmf13* genes from cDNA libraries. For example, the antibodies can be used to screen  $\lambda$ gt11 expression libraries, or the antibodies can be used for immunoscreening following hybrid selection and translation (see, for example, Ausubel (1995) at pages 6-12 to 6-16; Margolis *et al.*, "Screening  $\lambda$  expression libraries with antibody and protein probes," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), pages 1-14 (Oxford University Press 1995)).

As an alternative, a *Zsmf13* gene can be obtained by synthesizing nucleic acid molecules using mutually priming long oligonucleotides and the nucleotide sequences described herein (see, for example, Ausubel (1995) at pages 8-8 to 8-9). Established techniques using the polymerase chain reaction provide the ability to synthesize DNA molecules at least two kilobases in length (Adang *et al.*, *Plant Molec. Biol.* 21:1131 (1993), Bambot *et al.*, *PCR Methods and Applications* 2:266 (1993), Dillon *et al.*, "Use of the Polymerase Chain Reaction for the Rapid Construction of Synthetic Genes," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 263-268, (Humana Press, Inc. 1993), and Holowachuk *et al.*, *PCR Methods Appl.* 4:299 (1995)).

The nucleic acid molecules of the present invention can also be synthesized with "gene machines" using protocols such as the phosphoramidite method. If chemically-synthesized double stranded DNA is required for an application such as the synthesis of a gene or a gene fragment, then each complementary strand is made separately. The production of short genes (60 to 80 base pairs) is technically straightforward and can be accomplished by synthesizing the complementary strands and then annealing them. For the production of longer genes (>300 base pairs), however, special strategies may be required, because the coupling efficiency of each cycle during chemical DNA synthesis is seldom 100%. To overcome this problem, synthetic genes (double-stranded) are assembled in modular form from single-stranded fragments that are from 20 to 100 nucleotides in length. For reviews on polynucleotide synthesis, see, for example, Glick and Pasternak, *Molecular Biotechnology, Principles and Applications of Recombinant DNA* (ASM Press 1994), Itakura *et al.*, *Annu. Rev. Biochem.* 53:323 (1984), and Climie *et al.*, *Proc. Nat'l Acad. Sci. USA* 87:633 (1990).

The sequence of a *Zsmf13* cDNA or *Zsmf13* genomic fragment can be determined using standard methods. *Zsmf13* polynucleotide sequences disclosed herein can also be used as probes or primers to clone 5' non-coding regions of a *Zsmf13* gene.

Promoter elements from a *Zsmf13* gene can be used to direct the expression of heterologous genes in tissues of, for example, transgenic animals or patients treated with gene therapy. The identification of genomic fragments containing a *Zsmf13* promoter or regulatory element can be achieved using well-established techniques, such as deletion analysis (see, generally, Ausubel (1995)).

Cloning of 5' flanking sequences also facilitates production of *Zsmf13* proteins by "gene activation," as disclosed in U.S. Patent No. 5,641,670. Briefly, expression of an endogenous *Zsmf13* gene in a cell is altered by introducing into the *Zsmf13* locus a DNA construct comprising at least a targeting sequence, a regulatory sequence, an exon, and an unpaired splice donor site. The targeting sequence is a *Zsmf13* 5' non-coding sequence that permits homologous recombination of the construct with the endogenous *Zsmf13* locus, whereby the sequences within the construct become operably linked with the endogenous *Zsmf13* coding sequence. In this way, an endogenous *Zsmf13* promoter can be replaced or supplemented with other regulatory sequences to provide enhanced, tissue-specific, or otherwise regulated expression.

#### 4. *Production of Zsmf13Gene Variants*

The present invention provides a variety of nucleic acid molecules, including DNA and RNA molecules, which encode the *Zsmf13* polypeptides disclosed herein. Those skilled in the art will readily recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules. SEQ ID NO:3 is a degenerate nucleotide sequence that encompasses all nucleic acid molecules that encode the *Zsmf13* polypeptides of SEQ ID NO:2. Those skilled in the art will recognize that the degenerate sequence of SEQ ID NO:3 also provides all RNA sequences encoding SEQ ID NO:2, by substituting U for T. Thus, the present invention contemplates *Zsmf13* polypeptide-encoding nucleic acid molecules comprising nucleotide 168 to nucleotide 821 of SEQ ID NO:1, and their RNA equivalents.

Table 1 sets forth the one-letter codes used within SEQ ID NO:3 to denote degenerate nucleotide positions. "Resolutions" are the nucleotides denoted by a code letter. "Complement" indicates the code for the complementary nucleotide(s). For example, the code Y denotes either C or T, and its complement R denotes A or G, A being complementary to T, and G being complementary to C.

*Table 1*

Nucleotide	Resolution	Complement	Resolution
A	A	T	T
C	C	G	G
G	G	C	C
T	T	A	A
R	A G	Y	C T
Y	C T	R	A G
M	A C	K	G T
K	G T	M	A C
S	C G	S	C G
W	A T	W	A T
H	A C T	D	A G T
B	C G T	V	A C G
V	A C G	B	C G T
D	A G T	H	A C T
N	A C G T	N	A C G T

The degenerate codons used in SEQ ID NO:3, encompassing all possible  
5 codons for a given amino acid, are set forth in Table 2.

Table 2

Amino Acid	One Letter Code	Codons	Degenerate Codon
Cys	C	TGC TGT	TGY
Ser	S	AGC AGT TCA TCC TCG TCT	WSN
Thr	T	ACA ACC ACG ACT	ACN
Pro	P	CCA CCC CCG CCT	CCN
Ala	A	GCA GCC GCG GCT	GCN
Gly	G	GGA GGC GGG GGT	GGN
Asn	N	AAC AAT	AAY
Asp	D	GAC GAT	GAY
Glu	E	GAA GAG	GAR
Gln	Q	CAA CAG	CAR
His	H	CAC CAT	CAY
Arg	R	AGA AGG CGA CGC CGG CGT	MGN
Lys	K	AAA AAG	AAR
Met	M	ATG	ATG
Ile	I	ATA ATC ATT	ATH
Leu	L	CTA CTC CTG CTT TTA TTG	YTN
Val	V	GTA GTC GTG GTT	GTN
Phe	F	TTC TTT	TTY
Tyr	Y	TAC TAT	TAY
Trp	W	TGG	TGG
Ter	.	TAA TAG TGA	TRR
Asn Asp	B		RAY
Glu Gln	Z		SAR
Any	X		NNN

One of ordinary skill in the art will appreciate that some ambiguity is introduced in determining a degenerate codon, representative of all possible codons encoding an amino acid. For example, the degenerate codon for serine (WSN) can, in some circumstances, encode arginine (AGR), and the degenerate codon for arginine (MGN) can, in some circumstances, encode serine (AGY). A similar relationship exists between codons encoding phenylalanine and leucine. Thus, some polynucleotides encompassed by the degenerate sequence may encode variant amino acid sequences, but one of ordinary skill in the art can easily identify such variant sequences by reference to the amino acid sequence of SEQ ID NO:2. Variant sequences can be readily tested for functionality as described herein.

Different species can exhibit "preferential codon usage." In general, see, Grantham *et al.*, *Nuc. Acids Res.* 8:1893 (1980), Haas *et al.* *Curr. Biol.* 6:315 (1996), Wain-Hobson *et al.*, *Gene* 13:355 (1981), Grosjean and Fiers, *Gene* 18:199 (1982), Holm, *Nuc. Acids Res.* 14:3075 (1986), Ikemura, *J. Mol. Biol.* 158:573 (1982), Sharp and Matassi, *Curr. Opin. Genet. Dev.* 4:851 (1994), Kane, *Curr. Opin. Biotechnol.* 6:494 (1995), and Makrides, *Microbiol. Rev.* 60:512 (1996). As used herein, the term "preferential codon usage" or "preferential codons" is a term of art referring to protein translation codons that are most frequently used in cells of a certain species, thus favoring one or a few representatives of the possible codons encoding each amino acid (See Table 2). For example, the amino acid threonine (Thr) may be encoded by ACA, ACC, ACG, or ACT, but in mammalian cells ACC is the most commonly used codon; in other species, for example, insect cells, yeast, viruses or bacteria, different thr codons may be preferential. Preferential codons for a particular species can be introduced into the polynucleotides of the present invention by a variety of methods known in the art. Introduction of preferential codon sequences into recombinant DNA can, for example, enhance production of the protein by making protein translation more efficient within a particular cell type or species. Therefore, the degenerate codon sequence disclosed in SEQ ID NO:3 serves as a template for optimizing expression of polynucleotides in various cell types and species commonly used in the art and disclosed herein. Sequences containing preferential codons can be tested and optimized for expression in various species, and tested for functionality as disclosed herein.

The present invention further provides variant polypeptides and nucleic acid molecules that represent counterparts from other species (orthologs). These species include, but are not limited to mammalian, avian, amphibian, reptile, fish, insect and other vertebrate and invertebrate species. Of particular interest are Zsmf13 polypeptides from other mammalian species, including porcine, ovine, bovine, canine, feline, equine, and other primate polypeptides. Orthologs of human Zsmf13 can be

cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses *Zsmf13*. Suitable sources of mRNA can be identified by probing northern blots with probes  
5 designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line.

A *Zsmf13*-encoding cDNA molecule can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the disclosed sequences. A cDNA can also be  
10 cloned using the polymerase chain reaction with primers designed from the representative human *Zsmf13* sequences disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to *Zsmf13* polypeptide. Similar techniques can also be applied to the isolation of genomic clones.

Those skilled in the art will recognize that the sequence disclosed in  
15 SEQ ID NO:1 represents a single allele of human *Zsmf13*, and that allelic variation and alternative splicing are expected to occur. Allelic variants of this sequence can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures. Allelic variants of the nucleotide sequence shown in SEQ ID  
20 NO:1, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the present invention, as are proteins which are allelic variants of SEQ ID NO:2. cDNA molecules generated from alternatively spliced mRNAs, which retain the properties of the *Zsmf13* polypeptide are included within the scope of the present invention, as are polypeptides encoded by such  
25 cDNAs and mRNAs. Allelic variants and splice variants of these sequences can be cloned by probing cDNA or genomic libraries from different individuals or tissues according to standard procedures known in the art.

Within certain embodiments of the invention, the isolated nucleic acid molecules can hybridize under stringent conditions to nucleic acid molecules  
30 comprising nucleotide sequences disclosed herein. For example, such nucleic acid molecules can hybridize under stringent conditions to nucleic acid molecules consisting of the nucleotide sequence of SEQ ID NO:1, to nucleic acid molecules consisting of the nucleotide sequence of nucleotides 225 to 821 of SEQ ID NO:1, or to nucleic acid molecules consisting of a nucleotide sequence complementary to SEQ ID NO:1 or to  
35 nucleotides 225 to 821 of SEQ ID NO:1. In general, stringent conditions are selected to be about 5°C lower than the thermal melting point ( $T_m$ ) for the specific sequence at a

defined ionic strength and pH. The  $T_m$  is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe.

A pair of nucleic acid molecules, such as DNA-DNA, RNA-RNA and DNA-RNA, can hybridize if the nucleotide sequences have some degree of complementarity. Hybrids can tolerate mismatched base pairs in the double helix, but the stability of the hybrid is influenced by the degree of mismatch. The  $T_m$  of the mismatched hybrid decreases by 1°C for every 1-1.5% base pair mismatch. Varying the stringency of the hybridization conditions allows control over the degree of mismatch that will be present in the hybrid. The degree of stringency increases as the hybridization temperature increases and the ionic strength of the hybridization buffer decreases. Stringent hybridization conditions encompass temperatures of about 5-25°C below the  $T_m$  of the hybrid and a hybridization buffer having up to 1 M Na<sup>+</sup>. Higher degrees of stringency at lower temperatures can be achieved with the addition of formamide which reduces the  $T_m$  of the hybrid about 1°C for each 1% formamide in the buffer solution. Generally, such stringent conditions include temperatures of 20-70°C and a hybridization buffer containing up to 6x SSC and 0-50% formamide. A higher degree of stringency can be achieved at temperatures of from 40-70°C with a hybridization buffer having up to 4x SSC and from 0-50% formamide. Highly stringent conditions typically encompass temperatures of 42-70°C with a hybridization buffer having up to 1x SSC and 0-50% formamide. Different degrees of stringency can be used during hybridization and washing to achieve maximum specific binding to the target sequence. Typically, the washes following hybridization are performed at increasing degrees of stringency to remove non-hybridized polynucleotide probes from hybridized complexes.

The above conditions are meant to serve as a guide and it is well within the abilities of one skilled in the art to adapt these conditions for use with a particular polypeptide hybrid. The  $T_m$  for a specific target sequence is the temperature (under defined conditions) at which 50% of the target sequence will hybridize to a perfectly matched probe sequence. Those conditions which influence the  $T_m$  include, the size and base pair content of the polynucleotide probe, the ionic strength of the hybridization solution, and the presence of destabilizing agents in the hybridization solution. Numerous equations for calculating  $T_m$  are known in the art, and are specific for DNA, RNA and DNA-RNA hybrids and polynucleotide probe sequences of varying length (see, for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition (Cold Spring Harbor Press 1989); Ausubel *et al.*, (eds.), *Current Protocols in Molecular Biology* (John Wiley and Sons, Inc. 1987); Berger and Kimmel (eds.), *Guide to Molecular Cloning Techniques*, (Academic Press, Inc. 1987); and Wetmur,



*Crit. Rev. Biochem. Mol. Biol.* 26:227 (1990)). Sequence analysis software such as OLIGO 6.0 (LSR; Long Lake, MN) and *Primer Premier 4.0* (Premier Biosoft International; Palo Alto, CA), as well as sites on the Internet, are available tools for analyzing a given sequence and calculating  $T_m$  based on user defined criteria. Such programs can also analyze a given sequence under defined conditions and identify suitable probe sequences. Typically, hybridization of longer polynucleotide sequences, >50 base pairs, is performed at temperatures of about 20-25°C below the calculated  $T_m$ . For smaller probes, <50 base pairs, hybridization is typically carried out at the  $T_m$  or 5-10°C below. This allows for the maximum rate of hybridization for DNA-DNA and DNA-RNA hybrids.

The length of the polynucleotide sequence influences the rate and stability of hybrid formation. Smaller probe sequences, <50 base pairs, reach equilibrium with complementary sequences rapidly, but may form less stable hybrids. Incubation times of anywhere from minutes to hours can be used to achieve hybrid formation. Longer probe sequences come to equilibrium more slowly, but form more stable complexes even at lower temperatures. Incubations are allowed to proceed overnight or longer. Generally, incubations are carried out for a period equal to three times the calculated  $Cot$  time.  $Cot$  time, the time it takes for the polynucleotide sequences to reassociate, can be calculated for a particular sequence by methods known in the art.

The base pair composition of polynucleotide sequence will effect the thermal stability of the hybrid complex, thereby influencing the choice of hybridization temperature and the ionic strength of the hybridization buffer. A-T pairs are less stable than G-C pairs in aqueous solutions containing sodium chloride. Therefore, the higher the G-C content, the more stable the hybrid. Even distribution of G and C residues within the sequence also contribute positively to hybrid stability. In addition, the base pair composition can be manipulated to alter the  $T_m$  of a given sequence. For example, 5-methyldeoxycytidine can be substituted for deoxycytidine and 5-bromodeoxuridine can be substituted for thymidine to increase the  $T_m$ , whereas 7-deaz-2'-deoxyguanosine can be substituted for guanosine to reduce dependence on  $T_m$ .

The ionic concentration of the hybridization buffer also affects the stability of the hybrid. Hybridization buffers generally contain blocking agents such as Denhardt's solution (Sigma Chemical Co., St. Louis, Mo.), denatured salmon sperm DNA, tRNA, milk powders (BLOTTO), heparin or SDS, and a  $Na^+$  source, such as SSC (1x SSC: 0.15 M sodium chloride, 15 mM sodium citrate) or SSPE (1x SSPE: 1.8 M NaCl, 10 mM  $NaH_2PO_4$ , 1 mM EDTA, pH 7.7). By decreasing the ionic concentration of the buffer, the stability of the hybrid is increased. Typically, hybridization buffers

contain from between 10 mM - 1 M Na<sup>+</sup>. The addition of destabilizing or denaturing agents such as formamide, tetralkylammonium salts, guanidinium cations or thiocyanate cations to the hybridization solution will alter the T<sub>m</sub> of a hybrid. Typically, formamide is used at a concentration of up to 50% to allow incubations to be  
5 carried out at more convenient and lower temperatures. Formamide also acts to reduce non-specific background when using RNA probes.

As an illustration, a nucleic acid molecule encoding a variant Zsmf13 polypeptide can be hybridized with a nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO:1 (or its complement) at 42°C overnight in a solution  
10 comprising 50% formamide, 5xSSC (1xSSC: 0.15 M sodium chloride and 15 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution (100x Denhardt's solution: 2% (w/v) Ficoll 400, 2% (w/v) polyvinylpyrrolidone, and 2% (w/v) bovine serum albumin), 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA. One of skill in the art can devise variations of these hybridization  
15 conditions. For example, the hybridization mixture can be incubated at a higher temperature, such as about 65°C, in a solution that does not contain formamide. Moreover, premixed hybridization solutions are available (e.g., EXPRESSHYB Hybridization Solution from CLONTECH Laboratories, Inc.), and hybridization can be performed according to the manufacturer's instructions.

Following hybridization, the nucleic acid molecules can be washed to  
20 remove non-hybridized nucleic acid molecules under stringent conditions, or under highly stringent conditions. Typical stringent washing conditions include washing in a solution of 0.5x - 2x SSC with 0.1% sodium dodecyl sulfate (SDS) at 55 - 65°C. For example, certain nucleic acid molecules encoding a variant Zsmf13 polypeptide can  
25 remain hybridized with a nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO:1 (or its complement) following stringent washing conditions, in which the wash stringency is equivalent to 0.5x - 2x SSC with 0.1% SDS at 55 - 65°C, including 0.5x SSC with 0.1% SDS at 55°C, or 2xSSC with 0.1% SDS at 65°C. One of skill in the art can readily devise equivalent conditions, for example, by substituting SSPE for  
30 SSC in the wash solution.

Typical highly stringent washing conditions include washing in a solution of 0.1x - 0.2x SSC with 0.1% sodium dodecyl sulfate (SDS) at 50 - 65°C. As an illustration, particular nucleic acid molecules encoding a variant Zsmf13 polypeptide remain hybridized with a nucleic acid molecule consisting of the nucleotide sequence of  
35 SEQ ID NO:1 (or its complement) following highly stringent washing conditions, in which the wash stringency is equivalent to 0.1x - 0.2x SSC with 0.1% SDS at 50 -

65°C, including 0.1x SSC with 0.1% SDS at 50°C, or 0.2xSSC with 0.1% SDS at 65°C.

The present invention also provides isolated Zsmf13 polypeptides that have a substantially similar sequence identity to the polypeptides of SEQ ID NO:2, or their orthologs. The term "substantially similar sequence identity" is used herein to denote polypeptides having 85%, 90%, 95% or greater than 95% sequence identity to the sequences shown in SEQ ID NO:2, or their orthologs.

The present invention also contemplates Zsmf13 variant nucleic acid molecules that can be identified using two criteria: a determination of the similarity between the encoded polypeptide with the amino acid sequence of SEQ ID NO:2, and a hybridization assay, as described above. Such Zsmf13 variants include nucleic acid molecules (1) that remain hybridized with a nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO:1 (or its complement) following stringent washing conditions, in which the wash stringency is equivalent to 0.5x - 2x SSC with 0.1% SDS at 55 - 65°C, and (2) that encode a polypeptide having 85%, 90%, 95% or greater than 95% sequence identity to the amino acid sequence of SEQ ID NO:2. Alternatively, certain Zsmf13 variants can be characterized as nucleic acid molecules (1) that remain hybridized with a nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO:1 (or its complement) following highly stringent washing conditions, in which the wash stringency is equivalent to 0.1x - 0.2x SSC with 0.1% SDS at 50 - 65°C, and (2) that encode a polypeptide having 85%, 90%, 95% or greater than 95% sequence identity to the amino acid sequence of SEQ ID NO:2.

Percent sequence identity is determined by conventional methods. See, for example, Altschul *et al.*, *Bull. Math. Bio.* 48:603 (1986), and Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1992). Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "BLOSUM62" scoring matrix of Henikoff and Henikoff (*ibid.*) as shown in Table 3 (amino acids are indicated by the standard one-letter codes). The percent identity is then calculated as:  $\frac{[\text{Total number of identical matches}]}{[\text{length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences}]}(100)$ .

Table 3

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
N	-2	0	6																	
D	-2	-2	1	6																
C	0	-3	-3	-3	9															
Q	-1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-1	-3	-3	-4	-3	4											
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	0	-3	-1	4	

5

10

15

20

Those skilled in the art appreciate that there are many established algorithms available to align two amino acid sequences. The "FASTA" similarity search algorithm of Pearson and Lipman is a suitable protein alignment method for examining the level of identity shared by an amino acid sequence disclosed herein and the amino acid sequence of a putative Zsmf13 variant. The FASTA algorithm is described by Pearson and Lipman, *Proc. Nat'l Acad. Sci. USA* 85:2444 (1988), and by Pearson, *Meth. Enzymol.* 183:63 (1990).

Briefly, FASTA first characterizes sequence similarity by identifying regions shared by the query sequence (*e.g.*, SEQ ID NO:2) and a test sequence that have either the highest density of identities (if the ktup variable is 1) or pairs of identities (if ktup=2), without considering conservative amino acid substitutions, insertions, or deletions. The ten regions with the highest density of identities are then rescored by comparing the similarity of all paired amino acids using an amino acid substitution matrix, and the ends of the regions are "trimmed" to include only those residues that contribute to the highest score. If there are several regions with scores greater than the "cutoff" value (calculated by a predetermined formula based upon the length of the sequence and the ktup value), then the trimmed initial regions are examined to determine whether the regions can be joined to form an approximate alignment with gaps. Finally, the highest scoring regions of the two amino acid sequences are aligned using a modification of the Needleman-Wunsch-Sellers algorithm (Needleman and Wunsch, *J. Mol. Biol.* 48:444 (1970); Sellers, *SIAM J. Appl. Math.* 26:787 (1974)), which allows for amino acid insertions and deletions. Illustrative parameters for FASTA analysis are: ktup=1, gap opening penalty=10, gap extension penalty=1, and substitution matrix=BLOSUM62. These parameters can be introduced into a FASTA program by modifying the scoring matrix file ("SMATRIX"), as explained in Appendix 2 of Pearson, *Meth. Enzymol.* 183:63 (1990).

FASTA can also be used to determine the sequence identity of nucleic acid molecules using a ratio as disclosed above. For nucleotide sequence comparisons, the ktup value can range between one to six, preferably from three to six, and most preferably, three. The other parameters can be set as: gap opening penalty=10, and gap extension penalty=1.

The present invention includes nucleic acid molecules that encode a polypeptide having a conservative amino acid change, compared with the amino acid sequence of SEQ ID NO:2. That is, variants can be obtained that contain one or more amino acid substitutions of SEQ ID NO:2, in which an alkyl amino acid is substituted

for an alkyl amino acid in a Zsmf13 amino acid sequence, an aromatic amino acid is substituted for an aromatic amino acid in a Zsmf13 amino acid sequence, a sulfur-containing amino acid is substituted for a sulfur-containing amino acid in a Zsmf13 amino acid sequence, a hydroxy-containing amino acid is substituted for a hydroxy-containing amino acid in a Zsmf13 amino acid sequence, an acidic amino acid is substituted for an acidic amino acid in a Zsmf13 amino acid sequence, a basic amino acid is substituted for a basic amino acid in a Zsmf13 amino acid sequence, or a dibasic monocarboxylic amino acid is substituted for a dibasic monocarboxylic amino acid in a Zsmf13 amino acid sequence.

Among the common amino acids, for example, a "conservative amino acid substitution" is illustrated by a substitution among amino acids within each of the following groups: (1) glycine, alanine, valine, leucine, and isoleucine, (2) phenylalanine, tyrosine, and tryptophan, (3) serine and threonine, (4) aspartate and glutamate, (5) glutamine and asparagine, and (6) lysine, arginine and histidine.

The BLOSUM62 table is an amino acid substitution matrix derived from about 2,000 local multiple alignments of protein sequence segments, representing highly conserved regions of more than 500 groups of related proteins (Henikoff and Henikoff, *Proc. Nat'l Acad. Sci. USA* 89:10915 (1992)). Accordingly, the BLOSUM62 substitution frequencies can be used to define conservative amino acid substitutions that may be introduced into the amino acid sequences of the present invention. Although it is possible to design amino acid substitutions based solely upon chemical properties (as discussed above), the language "conservative amino acid substitution" can refer to a substitution represented by a BLOSUM62 value of greater than -1. For example, an amino acid substitution is conservative if the substitution is characterized by a BLOSUM62 value of 0, 1, 2, or 3. According to this system, conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 1 (e.g., 1, 2 or 3), while other conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 2 (e.g., 2 or 3).

Particular variants of Zsmf13 are characterized by having at least 70%, at least 80%, at least 85%, at least 90%, at least 95% or greater than 95% sequence identity to the corresponding amino acid sequence (i.e., SEQ ID NO:2), wherein the variation in amino acid sequence is due to one or more conservative amino acid substitutions.

Conservative amino acid changes in a Zsmf13 gene can be introduced by substituting nucleotides for the nucleotides recited in SEQ ID NO:1. Such "conservative amino acid" variants can be obtained, for example, by oligonucleotide-

directed mutagenesis, linker-scanning mutagenesis, mutagenesis using the polymerase chain reaction, and the like (see Ausubel (1995) at pages 8-10 to 8-22; and McPherson (ed.), *Directed Mutagenesis: A Practical Approach* (IRL Press 1991)). A variant Zsmf13 polypeptide can be identified by the ability to specifically bind anti-Zsmf13 antibodies.

The proteins of the present invention can also comprise non-naturally occurring amino acid residues. Non-naturally occurring amino acids include, without limitation, *trans*-3-methylproline, 2,4-methanoproline, *cis*-4-hydroxyproline, *trans*-4-hydroxyproline, *N*-methylglycine, *allo*-threonine, methylthreonine, hydroxyethylcysteine, hydroxyethylhomocysteine, nitroglutamine, homoglutamine, pipercolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, 3,3-dimethylproline, *tert*-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, and 4-fluorophenylalanine. Several methods are known in the art for incorporating non-naturally occurring amino acid residues into proteins. For example, an *in vitro* system can be employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations is typically carried out in a cell-free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson *et al.*, *J. Am. Chem. Soc.* 113:2722 (1991), Ellman *et al.*, *Methods Enzymol.* 202:301 (1991), Chung *et al.*, *Science* 259:806 (1993), and Chung *et al.*, *Proc. Nat'l Acad. Sci. USA* 90:10145 (1993).

In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs (Turcatti *et al.*, *J. Biol. Chem.* 271:19991 (1996)). Within a third method, *E. coli* cells are cultured in the absence of a natural amino acid that is to be replaced (*e.g.*, phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (*e.g.*, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the protein in place of its natural counterpart. See, Koide *et al.*, *Biochem.* 33:7470 (1994). Naturally occurring amino acid residues can be converted to non-naturally occurring species by *in vitro* chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, *Protein Sci.* 2:395 (1993)).

A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, non-naturally occurring amino acids, and unnatural amino acids may be substituted for Zsmf13 amino acid residues.

Amino acid sequence analysis indicates that a complete Zsmf13 polypeptide represents a human homologue of murine semaphorin G (Adams *et al.*, *Mech. Dev.* 57:33 (1996); GenBank Accession No. X97818; SEQ ID NO:5). Accordingly, particular Zsmf13 variants can be designed by modifying the human sequence to include one or more amino acid substitutions corresponding with the murine sequence. For example, Table 4 presents differences between SEQ ID NO:2 and the murine semaphorin G, which were identified following alignment of the amino acid sequences. Using the illustrative table, variants of the human semaphorin sequence can be devised by introducing one or more amino acid substitutions from the corresponding murine sequence. Alternatively, variant murine semaphorin sequences can be designed by introducing one or more amino acid substitutions from the corresponding human sequence. In this way, it is possible to obtain a "humanized" variant of the murine semaphorin sequence. Although Zsmf13 variants can be designed with any number of amino acid substitutions, certain variants will include at least about X amino acid substitutions, wherein X is selected from the group consisting of 2, 5, 10, 12, 15, 17, 20, 22, and 25.



Table 4

Amino Acid Position	Amino Acid	
	Zsmf13 (SEQ ID NO:2)	Murine Semaphorin G (SEQ ID NO:5)
3	leucine	valine
4	alanine	proline
9	valine	leucine
14	proline	serine
29	valine	isoleucine
30	serine	alanine
33	proline	serine
39	leucine	methionine
41	alanine	threonine
42	leucine	arginine
43	serine	arginine
44	lysine	glutamic acid
47	threonine	isoleucine
54	glutamine	lysine
58	serine	phenylalanine
65	alanine	valine
77	glycine	arginine
84	alanine	glycine
134	alanine	serine
149	methionine	valine
151	threonine	serine
214	proline	leucine
216	alanine	threonine
217	glutamic acid	alanine
218	phenylalanine	glutamine

5                    Essential amino acids in the polypeptides of the present invention can  
be identified according to procedures known in the art, such as site-directed  
mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science*  
244:1081 (1989), Bass *et al.*, *Proc. Nat'l Acad. Sci. USA* 88:4498 (1991), Coombs and  
Corey, "Site-Directed Mutagenesis and Protein Engineering," in *Proteins: Analysis*  
10 *and Design*, Angeletti (ed.), pages 259-311 (Academic Press, Inc. 1998)). In the latter  
technique, single alanine mutations are introduced at every residue in the molecule,  
and the resultant mutant molecules are tested for biological activity, such as the ability

to bind to an antibody, to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al.*, *J. Biol. Chem.* 271:4699 (1996).

The location of Zsmf13 receptor binding domains can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, *Science* 255:306 (1992), Smith *et al.*, *J. Mol. Biol.* 224:899 (1992), and Wlodaver *et al.*, *FEBS Lett.* 309:59 (1992). Moreover, Zsmf13 labeled with biotin or FITC can be used for expression cloning of Zsmf13 receptors.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (*Science* 241:53 (1988)) or Bowie and Sauer (*Proc. Nat'l Acad. Sci. USA* 86:2152 (1989)). Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (*e.g.*, Lowman *et al.*, *Biochem.* 30:10832 (1991), Ladner *et al.*, U.S. Patent No. 5,223,409, Huse, international publication No. WO 92/06204, and region-directed mutagenesis (Derbyshire *et al.*, *Gene* 46:145 (1986), and Ner *et al.*, *DNA* 7:127, (1988)).

Variants of the disclosed Zsmf13 nucleotide and polypeptide sequences can also be generated through DNA shuffling as disclosed by Stemmer, *Nature* 370:389 (1994), Stemmer, *Proc. Nat'l Acad. Sci. USA* 91:10747 (1994), and international publication No. WO 97/20078. Briefly, variant DNA molecules are generated by *in vitro* homologous recombination by random fragmentation of a parent DNA followed by reassembly using PCR, resulting in randomly introduced point mutations. This technique can be modified by using a family of parent DNA molecules, such as allelic variants or DNA molecules from different species, to introduce additional variability into the process. Selection or screening for the desired activity, followed by additional iterations of mutagenesis and assay provides for rapid "evolution" of sequences by selecting for desirable mutations while simultaneously selecting against detrimental changes.

Mutagenesis methods as disclosed herein can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides in host cells. Mutagenized DNA molecules that encode biologically active polypeptides, or polypeptides that bind with anti-Zsmf13 antibodies, can be

recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

5           The present invention also includes "functional fragments" of *Zsmf13* polypeptides and nucleic acid molecules encoding such functional fragments. Routine deletion analyses of nucleic acid molecules can be performed to obtain functional fragments of a nucleic acid molecule that encodes a *Zsmf13* polypeptide. As an illustration, DNA molecules having the nucleotide sequence of SEQ ID NO:1 can be  
10 digested with *Bal31* nuclease to obtain a series of nested deletions. The fragments are then inserted into expression vectors in proper reading frame, and the expressed polypeptides are isolated and tested for the ability to bind anti-*Zsmf13* antibodies. One alternative to exonuclease digestion is to use oligonucleotide-directed mutagenesis to introduce deletions or stop codons to specify production of a desired  
15 fragment. Alternatively, particular fragments of a *Zsmf13* gene can be synthesized using the polymerase chain reaction.

Methods for identifying functional domains are well-known to those of skill in the art. For example, studies on the truncation at either or both termini of interferons have been summarized by Horisberger and Di Marco, *Pharmac. Ther.*  
20 66:507 (1995). Moreover, standard techniques for functional analysis of proteins are described by, for example, Treuter *et al.*, *Molec. Gen. Genet.* 240:113 (1993), Content *et al.*, "Expression and preliminary deletion analysis of the 42 kDa 2-5A synthetase induced by human interferon," in *Biological Interferon Systems, Proceedings of ISIR-TNO Meeting on Interferon Systems*, Cantell (ed.), pages 65-72 (Nijhoff 1987),  
25 Herschman, "The EGF Receptor," in *Control of Animal Cell Proliferation, Vol. 1*, Boynton *et al.*, (eds.) pages 169-199 (Academic Press 1985), Coumailleau *et al.*, *J. Biol. Chem.* 270:29270 (1995); Fukunaga *et al.*, *J. Biol. Chem.* 270:25291 (1995); Yamaguchi *et al.*, *Biochem. Pharmacol.* 50:1295 (1995), and Meisel *et al.*, *Plant Molec. Biol.* 30:1 (1996).

30           The present invention also contemplates functional fragments of a *Zsmf13* gene that have amino acid changes, compared with the amino acid sequence of SEQ ID NO:2. A variant *Zsmf13* gene can be identified on the basis of structure by determining the level of identity with nucleotide and amino acid sequences of SEQ ID NOs:1 and 2, as discussed above. An alternative approach to identifying a variant  
35 gene on the basis of structure is to determine whether a nucleic acid molecule

encoding a potential variant *Zsmf13* gene can hybridize to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, as discussed above.

The present invention also provides polypeptide fragments or peptides comprising an epitope-bearing portion of a *Zsmf13* polypeptide described herein.  
5 Such fragments or peptides may comprise an "immunogenic epitope," which is a part of a protein that elicits an antibody response when the entire protein is used as an immunogen. Immunogenic epitope-bearing peptides can be identified using standard methods (see, for example, Geysen *et al.*, *Proc. Nat'l Acad. Sci. USA* 81:3998 (1983)).

In contrast, polypeptide fragments or peptides may comprise an  
10 "antigenic epitope," which is a region of a protein molecule to which an antibody can specifically bind. Certain epitopes consist of a linear or contiguous stretch of amino acids, and the antigenicity of such an epitope is not disrupted by denaturing agents. It is known in the art that relatively short synthetic peptides that can mimic epitopes of a protein can be used to stimulate the production of antibodies against the protein (see,  
15 for example, Sutcliffe *et al.*, *Science* 219:660 (1983)). Accordingly, antigenic epitope-bearing peptides and polypeptides of the present invention are useful to raise antibodies that bind with the polypeptides described herein.

Antigenic epitope-bearing peptides and polypeptides can contain at least four to ten amino acids, at least ten to fifteen amino acids, or about 15 to about  
20 30 amino acids of SEQ ID NO:2. Such epitope-bearing peptides and polypeptides can be produced by fragmenting a *Zsmf13* polypeptide, or by chemical peptide synthesis, as described herein. Moreover, epitopes can be selected by phage display of random peptide libraries (see, for example, Lane and Stephen, *Curr. Opin. Immunol.* 5:268 (1993), and Cortese *et al.*, *Curr. Opin. Biotechnol.* 7:616 (1996)). Standard methods  
25 for identifying epitopes and producing antibodies from small peptides that comprise an epitope are described, for example, by Mole, "Epitope Mapping," in *Methods in Molecular Biology*, Vol. 10, Manson (ed.), pages 105-116 (The Humana Press, Inc. 1992), Price, "Production and Characterization of Synthetic Peptide-Derived Antibodies," in *Monoclonal Antibodies: Production, Engineering, and Clinical*  
30 *Application*, Ritter and Ladyman (eds.), pages 60-84 (Cambridge University Press 1995), and Coligan *et al.* (eds.), *Current Protocols in Immunology*, pages 9.3.1 - 9.3.5 and pages 9.4.1 - 9.4.11 (John Wiley & Sons 1997).

Regardless of the particular nucleotide sequence of a variant *Zsmf13* gene, the gene encodes a polypeptide that may be characterized by its ability to bind  
35 specifically to an anti-*Zsmf13* antibody.

For any Zsmf13 polypeptide, including variants and fusion proteins, one of ordinary skill in the art can readily generate a fully degenerate polynucleotide sequence encoding that variant using the information set forth in Tables 1 and 2 above. Moreover, those of skill in the art can use standard software to devise Zsmf13 variants based upon the nucleotide and amino acid sequences described herein. Accordingly, the present invention includes a computer-readable medium encoded with a data structure that provides at least one of the following sequences: SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3. Suitable forms of computer-readable media include magnetic media and optically-readable media. Examples of magnetic media include a hard or fixed drive, a random access memory (RAM) chip, a floppy disk, digital linear tape (DLT), a disk cache, and a ZIP disk. Optically readable media are exemplified by compact discs (*e.g.*, CD-read only memory (ROM), CD-rewritable (RW), and CD-recordable), and digital versatile/video discs (DVD) (*e.g.*, DVD-ROM, DVD-RAM, and DVD+RW).

#### 5. ***Production of Zsmf13 Fusion Proteins***

Fusion proteins of Zsmf13 can be used to express Zsmf13 in a recombinant host, and to isolate expressed Zsmf13. One type of fusion protein comprises a peptide that guides a Zsmf13 polypeptide from a recombinant host cell. To direct a Zsmf13 polypeptide into the secretory pathway of a eukaryotic host cell, a secretory signal sequence (also known as a signal peptide, a leader sequence, prepro sequence or pre sequence) is provided in the Zsmf13 expression vector. While the secretory signal sequence may be derived from Zsmf13, a suitable signal sequence may also be derived from another secreted protein or synthesized *de novo*. The secretory signal sequence is operably linked to a Zsmf13-encoding sequence such that the two sequences are joined in the correct reading frame and positioned to direct the newly synthesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the nucleotide sequence encoding the polypeptide of interest, although certain secretory signal sequences may be positioned elsewhere in the nucleotide sequence of interest (see, *e.g.*, Welch *et al.*, U.S. Patent No. 5,037,743; Holland *et al.*, U.S. Patent No. 5,143,830).

Although the secretory signal sequence of Zsmf13 or another protein produced by mammalian cells (*e.g.*, tissue-type plasminogen activator signal sequence, as described, for example, in U.S. Patent No. 5,641,655) is useful for expression of Zsmf13 in recombinant mammalian hosts, a yeast signal sequence is preferred for expression in yeast cells. Examples of suitable yeast signal sequences are those

derived from yeast mating pheromone  $\alpha$ -factor (encoded by the *MF $\alpha$ 1* gene), invertase (encoded by the *SUC2* gene), or acid phosphatase (encoded by the *PHO5* gene). See, for example, Romanos *et al.*, "Expression of Cloned Genes in Yeast," in *DNA Cloning 2: A Practical Approach*, 2<sup>nd</sup> Edition, Glover and Hames (eds.), pages 123-167 (Oxford University Press 1995).

In bacterial cells, it is often desirable to express a heterologous protein as a fusion protein to decrease toxicity, increase stability, and to enhance recovery of the expressed protein. For example, Zsmf13 can be expressed as a fusion protein comprising a glutathione S-transferase polypeptide. Glutathione S-transferase fusion proteins are typically soluble, and easily purifiable from *E. coli* lysates on immobilized glutathione columns. In similar approaches, a Zsmf13 fusion protein comprising a maltose binding protein polypeptide can be isolated with an amylose resin column, while a fusion protein comprising the C-terminal end of a truncated Protein A gene can be purified using IgG-Sepharose. Established techniques for expressing a heterologous polypeptide as a fusion protein in a bacterial cell are described, for example, by Williams *et al.*, "Expression of Foreign Proteins in *E. coli* Using Plasmid Vectors and Purification of Specific Polyclonal Antibodies," in *DNA Cloning 2: A Practical Approach*, 2<sup>nd</sup> Edition, Glover and Hames (Eds.), pages 15-58 (Oxford University Press 1995). In addition, commercially available expression systems are available. For example, the PINPOINT Xa protein purification system (Promega Corporation; Madison, WI) provides a method for isolating a fusion protein comprising a polypeptide that becomes biotinylated during expression with a resin that comprises avidin.

Peptide tags that are useful for isolating heterologous polypeptides expressed by either prokaryotic or eukaryotic cells include polyHistidine tags (which have an affinity for nickel-chelating resin), *c-myc* tags, calmodulin binding protein (isolated with calmodulin affinity chromatography), substance P, the RYIRS tag (which binds with anti-RYIRS antibodies), the Glu-Glu tag, and the FLAG tag (which binds with anti-FLAG antibodies). See, for example, Luo *et al.*, *Arch. Biochem. Biophys.* 329:215 (1996), Morganti *et al.*, *Biotechnol. Appl. Biochem.* 23:67 (1996), and Zheng *et al.*, *Gene* 186:55 (1997). Nucleic acid molecules encoding such peptide tags are available, for example, from Sigma-Aldrich Corporation (St. Louis, MO).

Another form of fusion protein comprises a Zsmf13 polypeptide and an immunoglobulin heavy chain constant region, typically an F<sub>C</sub> fragment, which contains two constant region domains and a hinge region but lacks the variable region. As an illustration, Chang *et al.*, U.S. Patent No. 5,723,125, describe a fusion protein

comprising a human interferon and a human immunoglobulin Fc fragment. The C-terminal of the interferon is linked to the N-terminal of the Fc fragment by a peptide linker moiety. An example of a peptide linker is a peptide comprising primarily a T cell inert sequence, which is immunologically inert. An exemplary peptide linker has the amino acid sequence: GGS GG SGGGG SGGGG S (SEQ ID NO:4). In this fusion protein, a preferred Fc moiety is a human  $\gamma 4$  chain, which is stable in solution and has little or no complement activating activity. Accordingly, the present invention contemplates a Zsmf13 fusion protein that comprises a Zsmf13 polypeptide moiety and a human Fc fragment, wherein the C-terminus of the Zsmf13 polypeptide moiety is attached to the N-terminus of the Fc fragment via a peptide linker, such as a peptide consisting of the amino acid sequence of SEQ ID NO:4.

In another variation, a Zsmf13 fusion protein comprises an IgG sequence, a Zsmf13 polypeptide moiety covalently joined to the amino terminal end of the IgG sequence, and a signal peptide that is covalently joined to the amino terminal of the Zsmf13 polypeptide moiety, wherein the IgG sequence consists of the following elements in the following order: a hinge region, a CH<sub>2</sub> domain, and a CH<sub>3</sub> domain. Accordingly, the IgG sequence lacks a CH<sub>1</sub> domain. The Zsmf13 polypeptide moiety displays a Zsmf13 activity, such as the ability to bind with a Zsmf13 receptor. This general approach to producing fusion proteins that comprise both antibody and nonantibody portions has been described by LaRochelle *et al.*, EP 742830 (WO 95/21258).

Fusion proteins comprising a Zsmf13 polypeptide moiety and an Fc moiety can be used, for example, as an *in vitro* assay tool. For example, the presence of a Zsmf13 receptor in a biological sample can be detected using these Zsmf13-antibody fusion proteins, in which the Zsmf13 moiety is used to target the cognate receptor, and a macromolecule, such as Protein A or anti-Fc antibody, is used to detect the bound fusion protein-ligand complex. In addition, antibody-Zsmf13 fusion proteins, comprising antibody variable domains, are useful as therapeutic proteins, in which the antibody moiety binds with a target antigen, such as a tumor associated antigen.

Fusion proteins can be prepared by methods known to those skilled in the art by preparing each component of the fusion protein and chemically conjugating them. Alternatively, a polynucleotide encoding both components of the fusion protein in the proper reading frame can be generated using known techniques and expressed by the methods described herein. For example, part or all of a domain(s) conferring a biological function can be exchanged between Zsmf13 of the present invention with

The carrier can be a conventional organic or inorganic carrier. Examples of carriers include water, buffer solution, alcohol, propylene glycol, macrogol, sesame oil, corn oil, and the like.

## 10      **6.      *Production of Zsmf13 Polypeptides***

The polypeptides of the present invention, including full-length polypeptides, functional fragments, and fusion proteins, can be produced in recombinant host cells following conventional techniques. To express a *Zsmf13* gene, a nucleic acid molecule encoding the polypeptide must be operably linked to regulatory sequences that  
15      control transcriptional expression in an expression vector and then, introduced into a host cell. In addition to transcriptional regulatory sequences, such as promoters and enhancers, expression vectors can include translational regulatory sequences and a marker gene which is suitable for selection of cells that carry the expression vector.

Expression vectors that are suitable for production of a foreign protein  
20      in eukaryotic cells typically contain (1) prokaryotic DNA elements coding for a bacterial replication origin and an antibiotic resistance marker to provide for the growth and selection of the expression vector in a bacterial host; (2) eukaryotic DNA elements that control initiation of transcription, such as a promoter; and (3) DNA elements that control the processing of transcripts, such as a transcription  
25      termination/polyadenylation sequence. As discussed above, expression vectors can also include nucleotide sequences encoding a secretory sequence that directs the heterologous polypeptide into the secretory pathway of a host cell. For example, a *Zsmf13* expression vector may comprise a *Zsmf13* gene and a secretory sequence derived from a *Zsmf13* gene or another secreted gene.

*Zsmf13* proteins of the present invention may be expressed in  
30      mammalian cells. Examples of suitable mammalian host cells include African green monkey kidney cells (Vero; ATCC CRL 1587), human embryonic kidney cells (293-HEK; ATCC CRL 1573), baby hamster kidney cells (BHK-21, BHK-570; ATCC CRL 8544, ATCC CRL 10314), canine kidney cells (MDCK; ATCC CCL 34), Chinese  
35      hamster ovary cells (CHO-K1; ATCC CCL61; CHO DG44 [Chasin *et al.*, *Som. Cell. Molec. Genet.* 12:555 1986]), rat pituitary cells (GH1; ATCC CCL82), HeLa S3 cells



(ATCC CCL2.2), rat hepatoma cells (H-4-II-E; ATCC CRL 1548) SV40-transformed monkey kidney cells (COS-1; ATCC CRL 1650) and murine embryonic cells (NIH-3T3; ATCC CRL 1658).

For a mammalian host, the transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, simian virus, or the like, in which the regulatory signals are associated with a particular gene which has a high level of expression. Suitable transcriptional and translational regulatory sequences also can be obtained from mammalian genes, such as actin, collagen, myosin, and metallothionein genes.

Transcriptional regulatory sequences include a promoter region sufficient to direct the initiation of RNA synthesis. Suitable eukaryotic promoters include the promoter of the mouse *metallothionein I* gene (Hamer *et al.*, *J. Molec. Appl. Genet.* 1:273 (1982)), the TK promoter of *Herpes* virus (McKnight, *Cell* 31:355 (1982)), the SV40 early promoter (Benoist *et al.*, *Nature* 290:304 (1981)), the Rous sarcoma virus promoter (Gorman *et al.*, *Proc. Nat'l Acad. Sci. USA* 79:6777 (1982)), the cytomegalovirus promoter (Foecking *et al.*, *Gene* 45:101 (1980)), and the mouse mammary tumor virus promoter (see, generally, Etcheverry, "Expression of Engineered Proteins in Mammalian Cell Culture," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 163-181 (John Wiley & Sons, Inc. 1996)).

Alternatively, a prokaryotic promoter, such as the bacteriophage T3 RNA polymerase promoter, can be used to control *Zsmf13* gene expression in mammalian cells if the prokaryotic promoter is regulated by a eukaryotic promoter (Zhou *et al.*, *Mol. Cell. Biol.* 10:4529 (1990), and Kaufman *et al.*, *Nucl. Acids Res.* 19:4485 (1991)).

An expression vector can be introduced into host cells using a variety of standard techniques including calcium phosphate transfection, liposome-mediated transfection, microprojectile-mediated delivery, electroporation, and the like. The transfected cells can be selected and propagated to provide recombinant host cells that comprise the expression vector stably integrated in the host cell genome. Techniques for introducing vectors into eukaryotic cells and techniques for selecting such stable transformants using a dominant selectable marker are described, for example, by Ausubel (1995) and by Murray (ed.), *Gene Transfer and Expression Protocols* (Humana Press 1991).

For example, one suitable selectable marker is a gene that provides resistance to the antibiotic neomycin. In this case, selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems can

selected agent to select for cells that produce high levels of the products of the introduced genes. A suitable amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (*e.g.*, hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used. Alternatively, markers that introduce an altered phenotype, such as green fluorescent protein, or cell surface proteins such as CD4, CD8, Class I MHC, placental alkaline phosphatase may be used to sort transfected cells from untransfected cells by such means as FACS sorting or magnetic bead separation technology.

Zsmf13 polypeptides can also be produced by cultured mammalian cells using a viral delivery system. Exemplary viruses for this purpose include adenovirus, herpesvirus, vaccinia virus and adeno-associated virus (AAV). Adenovirus, a double-stranded DNA virus, is currently the best studied gene transfer vector for delivery of heterologous nucleic acid (for a review, see Becker *et al.*, *Meth. Cell Biol.* 43:161 (1994), and Douglas and Curiel, *Science & Medicine* 4:44 (1997)). Advantages of the adenovirus system include the accommodation of relatively large DNA inserts, the ability to grow to high-titer, the ability to infect a broad range of mammalian cell types, and flexibility that allows use with a large number of available vectors containing different promoters.

By deleting portions of the adenovirus genome, larger inserts (up to 7 kb) of heterologous DNA can be accommodated. These inserts can be incorporated into the viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. An option is to delete the essential *E1* gene from the viral vector, which results in the inability to replicate unless the *E1* gene is provided by the host cell. Adenovirus vector-infected human 293 cells (ATCC Nos. CRL-1573, 45504, 45505), for example, can be grown as adherent cells or in suspension culture at relatively high cell density to produce significant amounts of protein (see Garnier *et al.*, *Cytotechnol.* 15:145 (1994)).

Zsmf13 genes may also be expressed in other higher eukaryotic cells, such as avian, fungal, insect, yeast, or plant cells. The baculovirus system provides an efficient means to introduce cloned Zsmf13 genes into insect cells. Suitable expression vectors are based upon the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), and contain well-known promoters such as *Drosophila heat shock protein (hsp) 70* promoter, *Autographa californica nuclear polyhedrosis virus*

*immediate-early* gene promoter (*ie-1*) and the *delayed early 39K* promoter, baculovirus *p10* promoter, and the *Drosophila metallothionein* promoter. A second method of making recombinant baculovirus utilizes a transposon-based system described by Luckow (Luckow, *et al.*, *J. Virol.* 67:4566 (1993)). This system, which  
5 utilizes transfer vectors, is sold in the BAC-to-BAC kit (Life Technologies, Rockville, MD). This system utilizes a transfer vector, PFASTBAC (Life Technologies) containing a Tn7 transposon to move the DNA encoding the Zsmf13 polypeptide into a baculovirus genome maintained in *E. coli* as a large plasmid called a "bacmid." See, Hill-Perkins and Possee, *J. Gen. Virol.* 71:971 (1990), Bonning, *et al.*, *J. Gen. Virol.*  
10 75:1551 (1994), and Chazenbalk, and Rapoport, *J. Biol. Chem.* 270:1543 (1995). In addition, transfer vectors can include an in-frame fusion with DNA encoding an epitope tag at the C- or N-terminus of the expressed Zsmf13 polypeptide, for example, a Glu-Glu epitope tag (Grussenmeyer *et al.*, *Proc. Nat'l Acad. Sci.* 82:7952 (1985)). Using a technique known in the art, a transfer vector containing a *Zsmf13* gene is  
15 transformed into *E. coli*, and screened for bacmids which contain an interrupted *lacZ* gene indicative of recombinant baculovirus. The bacmid DNA containing the recombinant baculovirus genome is then isolated using common techniques.

The illustrative PFASTBAC vector can be modified to a considerable degree. For example, the polyhedrin promoter can be removed and substituted with  
20 the baculovirus basic protein promoter (also known as *Pcor*, p6.9 or MP promoter) which is expressed earlier in the baculovirus infection, and has been shown to be advantageous for expressing secreted proteins (see, for example, Hill-Perkins and Possee, *J. Gen. Virol.* 71:971 (1990), Bonning, *et al.*, *J. Gen. Virol.* 75:1551 (1994), and Chazenbalk and Rapoport, *J. Biol. Chem.* 270:1543 (1995). In such transfer  
25 vector constructs, a short or long version of the basic protein promoter can be used. Moreover, transfer vectors can be constructed which replace the native Zsmf13 secretory signal sequences with secretory signal sequences derived from insect proteins. For example, a secretory signal sequence from Ecdysteroid Glucosyltransferase (EGT), honey bee Melittin (Invitrogen Corporation; Carlsbad,  
30 CA), or baculovirus gp67 (PharMingen; San Diego, CA) can be used in constructs to replace the native Zsmf13 secretory signal sequence.

The recombinant virus or bacmid is used to transfect host cells. Suitable insect host cells include cell lines derived from IPLB-Sf-21, a *Spodoptera frugiperda* pupal ovarian cell line, such as Sf9 (ATCC CRL 1711), Sf21AE, and Sf21  
35 (Invitrogen Corporation; San Diego, CA), as well as *Drosophila* Schneider-2 cells, and the HIGH FIVEO cell line (Invitrogen) derived from *Trichoplusia ni* (U.S. Patent

No. 5,300,435). Commercially available serum-free media can be used to grow and to maintain the cells. Suitable media are Sf900 II™ (Life Technologies) or ESF 921™ (Expression Systems) for the Sf9 cells; and Ex-cello405™ (JRH Biosciences, Lenexa, KS) or Express FiveO™ (Life Technologies) for the *T. ni* cells. When recombinant virus is used, the cells are typically grown up from an inoculation density of approximately  $2-5 \times 10^5$  cells to a density of  $1-2 \times 10^6$  cells at which time a recombinant viral stock is added at a multiplicity of infection (MOI) of 0.1 to 10, more typically near 3.

Established techniques for producing recombinant proteins in baculovirus systems are provided by Bailey *et al.*, "Manipulation of Baculovirus Vectors," in *Methods in Molecular Biology, Volume 7: Gene Transfer and Expression Protocols*, Murray (ed.), pages 147-168 (The Humana Press, Inc. 1991), by Patel *et al.*, "The baculovirus expression system," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), pages 205-244 (Oxford University Press 1995), by Ausubel (1995) at pages 16-37 to 16-57, by Richardson (ed.), *Baculovirus Expression Protocols* (The Humana Press, Inc. 1995), and by Lucknow, "Insect Cell Expression Technology," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 183-218 (John Wiley & Sons, Inc. 1996).

Fungal cells, including yeast cells, can also be used to express the genes described herein. Yeast species of particular interest in this regard include *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Pichia methanolica*. Suitable promoters for expression in yeast include promoters from *GAL1* (galactose), *PGK* (phosphoglycerate kinase), *ADH* (alcohol dehydrogenase), *AOX1* (alcohol oxidase), *HIS4* (histidinol dehydrogenase), and the like. Many yeast cloning vectors have been designed and are readily available. These vectors include YIp-based vectors, such as YIp5, YRp vectors, such as YRp17, YEp vectors such as YEp13 and YCp vectors, such as YCp19. Methods for transforming *S. cerevisiae* cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311, Kawasaki *et al.*, U.S. Patent No. 4,931,373, Brake, U.S. Patent No. 4,870,008, Welch *et al.*, U.S. Patent No. 5,037,743, and Murray *et al.*, U.S. Patent No. 4,845,075. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (*e.g.*, leucine). A suitable vector system for use in *Saccharomyces cerevisiae* is the *POT1* vector system disclosed by Kawasaki *et al.* (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Additional suitable promoters and terminators for use in

yeast include those from glycolytic enzyme genes (see, *e.g.*, Kawasaki, U.S. Patent No. 4,599,311, Kingsman *et al.*, U.S. Patent No. 4,615,974, and Bitter, U.S. Patent No. 4,977,092) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446, 5,063,154, 5,139,936, and 4,661,454.

5 Transformation systems for other yeasts, including *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Ustilago maydis*, *Pichia pastoris*, *Pichia methanolica*, *Pichia guilliermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson *et al.*, *J. Gen. Microbiol.* 132:3459 (1986), and Cregg, U.S. Patent No. 4,882,279. *Aspergillus* cells  
10 may be utilized according to the methods of McKnight *et al.*, U.S. Patent No. 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino *et al.*, U.S. Patent No. 5,162,228. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533.

For example, the use of *Pichia methanolica* as host for the production  
15 of recombinant proteins is disclosed by Raymond, U.S. Patent No. 5,716,808, Raymond, U.S. Patent No. 5,736,383, Raymond *et al.*, *Yeast* 14:11-23 (1998), and in international publication Nos. WO 97/17450, WO 97/17451, WO 98/02536, and WO 98/02565. DNA molecules for use in transforming *P. methanolica* will commonly be prepared as double-stranded, circular plasmids, which can be linearized prior to  
20 transformation. For polypeptide production in *P. methanolica*, the promoter and terminator in the plasmid can be that of a *P. methanolica* gene, such as a *P. methanolica* alcohol utilization gene (*AUG1* or *AUG2*). Other useful promoters include those of the dihydroxyacetone synthase (DHAS), formate dehydrogenase (FMD), and catalase (CAT) genes. To facilitate integration of the DNA into the host  
25 chromosome, the entire expression segment of the plasmid can be flanked at both ends by host DNA sequences. A suitable selectable marker for use in *Pichia methanolica* is a *P. methanolica ADE2* gene, which encodes phosphoribosyl-5-aminoimidazole carboxylase (AIRC; EC 4.1.1.21), and which allows *ade2* host cells to grow in the absence of adenine. For large-scale, industrial processes where it is desirable to  
30 minimize the use of methanol, host cells can be used in which both methanol utilization genes (*AUG1* and *AUG2*) are deleted. For production of secreted proteins, host cells deficient in vacuolar protease genes (*PEP4* and *PRB1*) are useful. Electroporation is used to facilitate the introduction of a plasmid containing DNA encoding a polypeptide of interest into *P. methanolica* cells. *P. methanolica* cells can  
35 be transformed by electroporation using an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm, preferably about 3.75 kV/cm,

Agrobacterium-mediated transformation, electroporation, and the like. See, for example, Horsch *et al.*, *Science* 227:1229 (1985), Klein *et al.*, *Biotechnology* 10:268 (1992), and Miki *et al.*, "Procedures for Introducing Foreign DNA into Plants," in *Methods in Plant Molecular Biology and Biotechnology*, Glick *et al.* (eds.), pages 67-88 (CRC Press, 1993).

Alternatively, *Zsmf13* genes can be expressed in prokaryotic host cells. Suitable promoters that can be used to express *Zsmf13* polypeptides in a prokaryotic host are well-known to those of skill in the art and include promoters capable of recognizing the T4, T3, Sp6 and T7 polymerases, the P<sub>R</sub> and P<sub>L</sub> promoters of bacteriophage lambda, the *trp*, *recA*, heat shock, *lacUV5*, *tac*, *lpp-lacSpr*, *phoA*, and *lacZ* promoters of *E. coli*, promoters of *B. subtilis*, the promoters of the bacteriophages of *Bacillus*, *Streptomyces* promoters, the *int* promoter of bacteriophage lambda, the *bla* promoter of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene. Prokaryotic promoters have been reviewed by Glick, *J. Ind. Microbiol.* 1:277 (1987), Watson *et al.*, *Molecular Biology of the Gene*, 4th Ed. (Benjamin Cummins 1987), and by Ausubel *et al.* (1995).

Suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*. Suitable strains of *E. coli* include BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pLysE, DH1, DH4I, DH5, DH5I, DH5IF', DH5MCR, DH10B, DH10B/p3, DH11S, C600, HB101, JM101, JM105, JM109, JM110, K38, RR1, Y1088, Y1089, CSH18, ER1451, and ER1647 (see, for example, Brown (ed.), *Molecular Biology Labfax* (Academic Press 1991)). Suitable strains of *Bacillus subtilis* include BR151, YB886, MI119, MI120, and B170 (see, for example, Hardy, "Bacillus Cloning Methods," in *DNA Cloning: A Practical Approach*, Glover (ed.) (IRL Press 1985)).

When expressing a *Zsmf13* polypeptide in bacteria such as *E. coli*, the polypeptide may be retained in the cytoplasm, typically as insoluble granules, or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are recovered and denatured using, for example, guanidine isothiocyanate or urea. The denatured polypeptide can then be refolded and dimerized by diluting the denaturant, such as by dialysis against a solution of urea and a combination of reduced and oxidized glutathione, followed by

dialysis against a buffered saline solution. In the latter case, the polypeptide can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic shock) to release the contents of the periplasmic space and recovering the protein, thereby obviating the need for denaturation and refolding.

Methods for expressing proteins in prokaryotic hosts are well-known to those of skill in the art (see, for example, Williams *et al.*, "Expression of foreign proteins in *E. coli* using plasmid vectors and purification of specific polyclonal antibodies," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), page 15 (Oxford University Press 1995), Ward *et al.*, "Genetic Manipulation and Expression of Antibodies," in *Monoclonal Antibodies: Principles and Applications*, page 137 (Wiley-Liss, Inc. 1995), and Georgiou, "Expression of Proteins in Bacteria," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), page 101 (John Wiley & Sons, Inc. 1996)).

Standard methods for introducing expression vectors into bacterial, yeast, insect, and plant cells are provided, for example, by Ausubel (1995).

General methods for expressing and recovering foreign protein produced by a mammalian cell system are provided by, for example, Etcheverry, "Expression of Engineered Proteins in Mammalian Cell Culture," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 163 (Wiley-Liss, Inc. 1996). Standard techniques for recovering protein produced by a bacterial system is provided by, for example, Grisshammer *et al.*, "Purification of over-produced proteins from *E. coli* cells," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), pages 59-92 (Oxford University Press 1995). Established methods for isolating recombinant proteins from a baculovirus system are described by Richardson (ed.), *Baculovirus Expression Protocols* (The Humana Press, Inc. 1995).

As an alternative, polypeptides of the present invention can be synthesized by exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. These synthesis methods are well-known to those of skill in the art (see, for example, Merrifield, *J. Am. Chem. Soc.* 85:2149 (1963), Stewart *et al.*, "Solid Phase Peptide Synthesis" (2nd Edition), (Pierce Chemical Co. 1984), Bayer and Rapp, *Chem. Pept. Prot.* 3:3 (1986), Atherton *et al.*, *Solid Phase Peptide Synthesis: A Practical Approach* (IRL Press 1989), Fields and Colowick, "Solid-Phase Peptide Synthesis," *Methods in Enzymology Volume 289* (Academic Press 1997), and Lloyd-Williams *et al.*, *Chemical Approaches to the Synthesis of Peptides and Proteins* (CRC Press, Inc. 1997)). Variations in total

chemical synthesis strategies, such as "native chemical ligation" and "expressed protein ligation" are also standard (see, for example, Dawson *et al.*, *Science* 266:776 (1994), Hackeng *et al.*, *Proc. Nat'l Acad. Sci. USA* 94:7845 (1997), Dawson, *Methods Enzymol.* 287: 34 (1997), Muir *et al.*, *Proc. Nat'l Acad. Sci. USA* 95:6705 (1998), and Severinov and Muir, *J. Biol. Chem.* 273:16205 (1998)).

Peptides and polypeptides of the present invention comprise at least six, at least nine, or at least 15 contiguous amino acid residues of SEQ ID NO:2, or the following sections of SEQ ID NO:2: amino acid residues 20 to 218, amino acid residues 20 to 132, or amino acid residues 200 to 218. Illustrative amino acid sequences comprise the following amino acid residues of SEQ ID NO:2: 20 to 34, 35 to 49, 50 to 64, 65 to 79, 80 to 94, 134 to 148, 149 to 164, and 204 to 218. Within certain embodiments of the invention, the polypeptides comprise 20, 30, 40, 50, 100, or more contiguous residues of the amino acid sequence of SEQ ID NO:2, amino acid residues 20 to 218 of SEQ ID NO:2, or amino acid residues 20 to 132 of SEQ ID NO:2. Nucleic acid molecules encoding such peptides and polypeptides are useful as polymerase chain reaction primers and probes.

## **7. Isolation of Zsmf13 Polypeptides**

The polypeptides of the present invention can be purified to at least about 80% purity, to at least about 90% purity, to at least about 95% purity, or greater than 95% purity with respect to contaminating macromolecules, particularly other proteins and nucleic acids, and free of infectious and pyrogenic agents. The polypeptides of the present invention may also be purified to a pharmaceutically pure state, which is greater than 99.9% pure. In certain preparations, a purified polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin.

Fractionation and/or conventional purification methods can be used to obtain preparations of Zsmf13 purified from natural sources (*e.g.*, adrenal tumor tissue), and recombinant Zsmf13 polypeptides and fusion Zsmf13 polypeptides purified from recombinant host cells. In general, ammonium sulfate precipitation and acid or chaotrope extraction may be used for fractionation of samples. Exemplary purification steps may include hydroxyapatite, size exclusion, FPLC and reverse-phase high performance liquid chromatography. Suitable chromatographic media include derivatized dextrans, agarose, cellulose, polyacrylamide, specialty silicas, and the like. PEI, DEAE, QAE and Q derivatives are preferred. Exemplary chromatographic media include those media derivatized with phenyl, butyl, or octyl groups, such as Phenyl-



Sepharose FF (Pharmacia), Toyopearl butyl 650 (Toso Haas, Montgomeryville, PA), Octyl-Sepharose (Pharmacia) and the like; or polyacrylic resins, such as Amberchrom CG 71 (Toso Haas) and the like. Suitable solid supports include glass beads, silica-based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene beads, cross-linked polyacrylamide resins and the like that are insoluble under the conditions in which they are to be used. These supports may be modified with reactive groups that allow attachment of proteins by amino groups, carboxyl groups, sulfhydryl groups, hydroxyl groups and/or carbohydrate moieties.

Examples of coupling chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, hydrazide activation, and carboxyl and amino derivatives for carbodiimide coupling chemistries. These and other solid media are well known and widely used in the art, and are available from commercial suppliers. Selection of a particular method for polypeptide isolation and purification is a matter of routine design and is determined in part by the properties of the chosen support. See, for example, *Affinity Chromatography: Principles & Methods* (Pharmacia LKB Biotechnology 1988), and Doonan, *Protein Purification Protocols* (The Humana Press 1996).

Additional variations in Zsmf13 isolation and purification can be devised by those of skill in the art. For example, anti-Zsmf13 antibodies, obtained as described below, can be used to isolate large quantities of protein by immunoaffinity purification. Moreover, methods for binding receptors, such as Zsmf13, to ligand polypeptides bound to support media are well known in the art.

The polypeptides of the present invention can also be isolated by exploitation of particular properties. For example, immobilized metal ion adsorption (IMAC) chromatography can be used to purify histidine-rich proteins, including those comprising polyhistidine tags. Briefly, a gel is first charged with divalent metal ions to form a chelate (Sulkowski, *Trends in Biochem. Sci.* 10:1 (1985)). Histidine-rich proteins will be adsorbed to this matrix with differing affinities, depending upon the metal ion used, and will be eluted by competitive elution, lowering the pH, or use of strong chelating agents. Other methods of purification include purification of glycosylated proteins by lectin affinity chromatography and ion exchange chromatography (M. Deutscher, (ed.), *Meth. Enzymol.* 182:529 (1990)). Within additional embodiments of the invention, a fusion of the polypeptide of interest and an affinity tag (e.g., maltose-binding protein, an immunoglobulin domain) may be constructed to facilitate purification.

Zsmf13 polypeptides or fragments thereof may also be prepared through chemical synthesis, as described above. Zsmf13 polypeptides may be monomers or multimers; glycosylated or non-glycosylated; pegylated or non-pegylated; and may or may not include an initial methionine amino acid residue.

5

### 8. *Zsmf13 Analogs and the Zsmf13 Receptor*

As described above, the disclosed polypeptides can be used to construct Zsmf13 variants. These polypeptides can be used to identify Zsmf13 analogs. One type of Zsmf13 analog mimics Zsmf13 by binding with a Zsmf13 receptor. Such an analog is considered to be a Zsmf13 agonist if the binding of the analog with a Zsmf13 receptor stimulates a response by a cell that expresses the receptor. On the other hand, a Zsmf13 analog that binds with a Zsmf13 receptor, but does not stimulate a cellular response, may be a Zsmf13 antagonist. Such an antagonist may diminish Zsmf13 or Zsmf13 agonist activity, for example, by a competitive or non-competitive binding of the antagonist to the Zsmf13 receptor.

One general class of Zsmf13 analogs are agonists or antagonists having an amino acid sequence that is a mutation of the amino acid sequences disclosed herein. Another general class of Zsmf13 analogs is provided by anti-idiotypic antibodies, and fragments thereof, as described below. Moreover, recombinant antibodies comprising anti-idiotypic variable domains can be used as analogs (see, for example, Monfardini *et al.*, *Proc. Assoc. Am. Physicians* 108:420 (1996)). Since the variable domains of anti-idiotypic Zsmf13 antibodies mimic Zsmf13, these domains can provide either Zsmf13 agonist or antagonist activity. As an illustration, Lim and Langer, *J. Interferon Res.* 13:295 (1993), describe anti-idiotypic interferon- $\alpha$  antibodies that have the properties of either interferon- $\alpha$  agonists or antagonists.

A third approach to identifying Zsmf13 analogs is provided by the use of combinatorial libraries. Methods for constructing and screening phage display and other combinatorial libraries are provided, for example, by Kay *et al.*, *Phage Display of Peptides and Proteins* (Academic Press 1996), Verdine, U.S. Patent No. 5,783,384, Kay, *et. al.*, U.S. Patent No. 5,747,334, and Kauffman *et al.*, U.S. Patent No. 5,723,323.

The Zsmf13, its agonists and antagonists are valuable in both *in vivo* and *in vitro* uses. For example, Zsmf13 or an agonist can be used as a component of defined cell culture media, alone or in combination with other bioactive agents, to replace serum that is commonly used in cell culture.

35

Antagonists are also useful as research reagents for characterizing sites of interaction between a Zsmf13 and its receptor. In a therapeutic setting, pharmaceutical compositions comprising Zsmf13 antagonists can be used to inhibit Zsmf13 activity.

5           Semaphorins can direct neurite growth and neuronal system organization. Accordingly, Zsmf13 polypeptides, agonists, and antagonists can be used to modulate neurite growth and development of nervous system structures *in vivo* or in *in vitro* cell culture systems.

10           Zsmf13 polypeptides can be used to identify and to isolate Zsmf13 receptors. For example, proteins and peptides of the present invention can be immobilized on a column and used to bind receptors from a biological sample that is run over the column (Hermanson *et al.* (eds.), *Immobilized Affinity Ligand Techniques*, pages 195-202 (Academic Press 1992)). As a receptor ligand, the activity of Zsmf13 can be measured by a silicon-based biosensor microphysiometer, which  
15           measures the extracellular acidification rate or proton excretion associated with receptor binding and subsequent cellular responses. An exemplary device is the CYTOSENSOR Microphysiometer manufactured by Molecular Devices Corp. (Sunnyvale, CA). A variety of cellular responses, such as cell proliferation, ion transport, energy production, inflammatory response, regulatory and receptor  
20           activation, and the like, can be measured by this method (see, for example, McConnell *et al.*, *Science* 257:1906 (1992), Pitchford *et al.*, *Meth. Enzymol.* 228:84 (1997), Arimilli *et al.*, *J. Immunol. Meth.* 212:49 (1998), and Van Liefde *et al.*, *Eur. J. Pharmacol.* 346:87 (1998)). Moreover, the microphysiometer can be used for assaying adherent or non-adherent eukaryotic or prokaryotic cells.

25           Since energy metabolism is coupled with the use of cellular ATP, any event which alters cellular ATP levels, such as receptor activation and the initiation of signal transduction, will cause a change in cellular acid section. By measuring extracellular acidification changes in cell media over time, therefore, the microphysiometer directly measures cellular responses to various stimuli, including  
30           Zsmf13, its agonists, or antagonists. The microphysiometer can be used to measure responses of a Zsmf13 responsive eukaryotic cell, compared to a control eukaryotic cell that does not respond to Zsmf13 polypeptide. Zsmf13 responsive eukaryotic cells comprise cells into which a receptor for Zsmf13 has been transfected to create a cell that is responsive to Zsmf13, or cells that are naturally responsive to Zsmf13. Zsmf13  
35           modulated cellular responses are measured by a change (*e.g.*, an increase or decrease

in extracellular acidification) in the response of cells exposed to Zsmf13, compared with control cells that have not been exposed to Zsmf13.

Accordingly, a microphysiometer can be used to identify cells, tissues, or cell lines which respond to a Zsmf13 stimulated pathway, and which express a functional Zsmf13 receptor. As an illustration, cells that express a functional Zsmf13 receptor can be identified by (a) providing test cells, (b) incubating a first portion of the test cells in the absence of Zsmf13, (c) incubating a second portion of the test cells in the presence of Zsmf13, and (d) detecting a change (*e.g.*, an increase or decrease in extracellular acidification rate, as measured by a microphysiometer) in a cellular response of the second portion of the test cells, as compared to the first portion of the test cells, wherein such a change in cellular response indicates that the test cells express a functional Zsmf13 receptor. An additional negative control may be included in which a portion of the test cells is incubated with Zsmf13 and an anti-Zsmf13 antibody to inhibit the binding of Zsmf13 with its cognate receptor.

Zsmf13, its analogs, and anti-idiotypic Zsmf13 antibodies can be used to identify and to isolate Zsmf13 receptors. For example, proteins and peptides of the present invention can be immobilized on a column and used to bind receptor proteins from membrane preparations that are run over the column (Hermanson *et al.* (eds.), *Immobilized Affinity Ligand Techniques*, pages 195-202 (Academic Press 1992)). Radiolabeled or affinity labeled Zsmf13 polypeptides can also be used to identify or to localize Zsmf13 receptors in a biological sample (see, for example, Deutscher (ed.), *Methods in Enzymol.*, vol. 182, pages 721-37 (Academic Press 1990); Brunner *et al.*, *Ann. Rev. Biochem.* 62:483 (1993); Fedan *et al.*, *Biochem. Pharmacol.* 33:1167 (1984)). Also see, Varthakavi and Minocha, *J. Gen. Virol.* 77:1875 (1996), who describe the use of anti-idiotypic antibodies for receptor identification.

In addition, a solid phase system can be used to identify a Zsmf13 receptor, or an agonist or antagonist of a Zsmf13 receptor. For example, a Zsmf13 polypeptide or Zsmf13 fusion protein can be immobilized onto the surface of a receptor chip of a commercially available biosensor instrument (BIAcore, Biacore AB; Uppsala, Sweden). The use of this instrument is disclosed, for example, by Karlsson, *Immunol. Methods* 145:229 (1991), and Cunningham and Wells, *J. Mol. Biol.* 234:554 (1993).

Zsmf13 receptor binding domains can be further characterized by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids of Zsmf13 agonists.

See, for example, de Vos *et al.*, *Science* 255:306 (1992), Smith *et al.*, *J. Mol. Biol.* 224:899 (1992), and Wlodaver *et al.*, *FEBS Lett.* 309:59 (1992).

In addition to the uses described above, polynucleotides and polypeptides of the present invention are useful as educational tools in laboratory practicum kits for courses related to genetics and molecular biology, protein chemistry, and antibody production and analysis. Due to its unique polynucleotide and polypeptide sequences, molecules of Zsmf13 can be used as standards or as "unknowns" for testing purposes. For example, Zsmf13 polynucleotides can be used as an aid, such as, for example, to teach a student how to prepare expression constructs for bacterial, viral, or mammalian expression, including fusion constructs, wherein Zsmf13 is the gene to be expressed; for determining the restriction endonuclease cleavage sites of the polynucleotides; determining mRNA and DNA localization of Zsmf13 polynucleotides in tissues (*i.e.*, by northern and Southern blotting as well as polymerase chain reaction); and for identifying related polynucleotides and polypeptides by nucleic acid hybridization. As an illustration, students will find that *Pvu*II digestion of a nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO:1 provides three fragments of about 282 base pairs, 487 base pairs, and 112 base pairs, and that *Sma*I digestion yields fragments of about 69 base pairs, 295 base pairs, and 457 base pairs.

Zsmf13 polypeptides can be used as an aid to teach preparation of antibodies; identifying proteins by western blotting; protein purification; determining the weight of expressed Zsmf13 polypeptides as a ratio to total protein expressed; identifying peptide cleavage sites; coupling amino and carboxyl terminal tags; amino acid sequence analysis, as well as, but not limited to monitoring biological activities of both the native and tagged protein (*i.e.*, protease inhibition) *in vitro* and *in vivo*. For example, students will find that digestion of unglycosylated Zsmf13 with cyanogen bromide yields four fragments having approximate molecular weights of 148, 15212, 926, and 7451, whereas digestion of unglycosylated Zsmf13 with BNPS or NCS/urea yields fragments having approximate molecular weights of 5958, 5314, and 12449.

Zsmf13 polypeptides can also be used to teach analytical skills such as mass spectrometry, circular dichroism, to determine conformation, especially of the four alpha helices, x-ray crystallography to determine the three-dimensional structure in atomic detail, nuclear magnetic resonance spectroscopy to reveal the structure of proteins in solution. For example, a kit containing the Zsmf13 can be given to the student to analyze. Since the amino acid sequence would be known by the instructor, the protein can be given to the student as a test to determine the skills or develop the

skills of the student, the instructor would then know whether or not the student has correctly analyzed the polypeptide. Since every polypeptide is unique, the educational utility of Zsmf13 would be unique unto itself.

5 The antibodies which bind specifically to Zsmf13 can be used as a teaching aid to instruct students how to prepare affinity chromatography columns to purify Zsmf13, cloning and sequencing the polynucleotide that encodes an antibody and thus as a practicum for teaching a student how to design humanized antibodies. The *Zsmf13* gene, polypeptide, or antibody would then be packaged by reagent companies and sold to educational institutions so that the students gain skill in art of molecular biology. Because each gene and protein is unique, each gene and protein creates unique challenges and learning experiences for students in a lab practicum. Such educational kits containing the *Zsmf13* gene, polypeptide, or antibody are considered within the scope of the present invention.

## 15 9. *Production of Antibodies to Zsmf13 Proteins*

Antibodies to Zsmf13 can be obtained, for example, using the product of a Zsmf13 expression vector or Zsmf13 isolated from a natural source as an antigen. Particularly useful anti-Zsmf13 antibodies "bind specifically" with Zsmf13. Antibodies are considered to be specifically binding if the antibodies exhibit at least one of the following two properties: (1) antibodies bind to Zsmf13 with a threshold level of binding activity, and (2) antibodies do not significantly cross-react with polypeptides related to Zsmf13, such as known semaphorins.

With regard to the first characteristic, antibodies specifically bind if they bind to a Zsmf13 polypeptide, peptide or epitope with a binding affinity ( $K_d$ ) of  $10^6 M^{-1}$  or greater, preferably  $10^7 M^{-1}$  or greater, more preferably  $10^8 M^{-1}$  or greater, and most preferably  $10^9 M^{-1}$  or greater. The binding affinity of an antibody can be readily determined by one of ordinary skill in the art, for example, by Scatchard analysis (Scatchard, *Ann. NY Acad. Sci.* 51:660 (1949)). With regard to the second characteristic, antibodies do not significantly cross-react with related polypeptide molecules, for example, if they detect Zsmf13, but not known related polypeptides using a standard Western blot analysis. Examples of known related polypeptides are semaphorin orthologs and semaphorin proteins from the same species that are members of a protein family. As an illustration, an anti-Zsmf13 antibody binds specifically to Zsmf13 if that antibody does not significantly cross-react with murine semaphorin G.

Anti-Zsmf13 antibodies can be produced using antigenic Zsmf13 epitope-bearing peptides and polypeptides. Antigenic epitope-bearing peptides and polypeptides of the present invention contain a sequence of at least four, preferably between 15 to about 30 amino acids contained within SEQ ID NO:2. However, peptides or polypeptides comprising a larger portion of an amino acid sequence of the invention, containing from 30 to 50 amino acids, or any length up to and including the entire amino acid sequence of a polypeptide of the invention, also are useful for inducing antibodies that bind with Zsmf13. It is desirable that the amino acid sequence of the epitope-bearing peptide is selected to provide substantial solubility in aqueous solvents (*i.e.*, the sequence includes relatively hydrophilic residues, while hydrophobic residues are preferably avoided). Moreover, amino acid sequences containing proline residues may be also be desirable for antibody production.

As an illustration, potential antigenic sites in Zsmf13 were identified using the Jameson-Wolf method, Jameson and Wolf, *CABIOS* 4:181, (1988), as implemented by the PROTEAN program (version 3.14) of LASERGENE (DNASTAR; Madison, WI). Default parameters were used in this analysis.

The Jameson-Wolf method predicts potential antigenic determinants by combining six major subroutines for protein structural prediction. Briefly, the Hopp-Woods method, Hopp *et al.*, *Proc. Nat'l Acad. Sci. USA* 78:3824 (1981), was first used to identify amino acid sequences representing areas of greatest local hydrophilicity (parameter: seven residues averaged). In the second step, Emini's method, Emini *et al.*, *J. Virology* 55:836 (1985), was used to calculate surface probabilities (parameter: surface decision threshold (0.6) = 1). Third, the Karplus-Schultz method, Karplus and Schultz, *Naturwissenschaften* 72:212 (1985), was used to predict backbone chain flexibility (parameter: flexibility threshold (0.2) = 1). In the fourth and fifth steps of the analysis, secondary structure predictions were applied to the data using the methods of Chou-Fasman, Chou, "Prediction of Protein Structural Classes from Amino Acid Composition," in *Prediction of Protein Structure and the Principles of Protein Conformation*, Fasman (ed.), pages 549-586 (Plenum Press 1990), and Garnier-Robson, Garnier *et al.*, *J. Mol. Biol.* 120:97 (1978) (Chou-Fasman parameters: conformation table = 64 proteins;  $\alpha$  region threshold = 103;  $\beta$  region threshold = 105; Garnier-Robson parameters:  $\alpha$  and  $\beta$  decision constants = 0). In the sixth subroutine, flexibility parameters and hydropathy/solvent accessibility factors were combined to determine a surface contour value, designated as the "antigenic index." Finally, a peak broadening function was applied to the antigenic index, which broadens major surface peaks by adding 20, 40, 60, or 80% of the respective peak

value to account for additional free energy derived from the mobility of surface regions relative to interior regions. This calculation was not applied, however, to any major peak that resides in a helical region, since helical regions tend to be less flexible.

5           The results of this analysis indicated that suitable antigenic molecules include the following segments of the amino acid sequence of SEQ ID NO:2: amino acids 27 to 35 ("antigenic molecule 1"), amino acids 43 to 48 ("antigenic molecule 2"), amino acids 63 to 68 ("antigenic molecule 3"), and amino acids 212 to 218 ("antigenic molecule 4"). The present invention contemplates the use of any one of  
10           antigenic molecules 1 to 4 to generate antibodies to Zsmf13. The present invention also contemplates polypeptides comprising at least one of antigenic molecules 1 to 4.

          Polyclonal antibodies to recombinant Zsmf13 protein or to Zsmf13 isolated from natural sources can be prepared using methods well-known to those of skill in the art. See, for example, Green *et al.*, "Production of Polyclonal Antisera," in  
15           *Immunochemical Protocols* (Manson, ed.), pages 1-5 (Humana Press 1992), and Williams *et al.*, "Expression of foreign proteins in *E. coli* using plasmid vectors and purification of specific polyclonal antibodies," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), page 15 (Oxford University Press 1995). The immunogenicity of a Zsmf13 polypeptide can be increased through the use of an  
20           adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of Zsmf13 or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like," such portion may be  
25           advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

          Although polyclonal antibodies are typically raised in animals such as horses, cows, dogs, chicken, rats, mice, rabbits, guinea pigs, goats, or sheep, an anti-  
30           Zsmf13 antibody of the present invention may also be derived from a subhuman primate antibody. General techniques for raising diagnostically and therapeutically useful antibodies in baboons may be found, for example, in Goldenberg *et al.*, international patent publication No. WO 91/11465, and in Losman *et al.*, *Int. J. Cancer* 46:310 (1990).

35           Alternatively, monoclonal anti-Zsmf13 antibodies can be generated. Rodent monoclonal antibodies to specific antigens may be obtained by methods



known to those skilled in the art (see, for example, Kohler *et al.*, *Nature* 256:495 (1975), Coligan *et al.* (eds.), *Current Protocols in Immunology*, Vol. 1, pages 2.5.1-2.6.7 (John Wiley & Sons 1991) ["Coligan"], Picklesley *et al.*, "Production of monoclonal antibodies against proteins expressed in *E. coli*," in *DNA Cloning 2: Expression Systems*, 2nd Edition, Glover *et al.* (eds.), page 93 (Oxford University Press 1995)).

Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising a Zsmf13 gene product, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B-lymphocytes, fusing the B-lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones which produce antibodies to the antigen, culturing the clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures.

In addition, an anti-Zsmf13 antibody of the present invention may be derived from a human monoclonal antibody. Human monoclonal antibodies are obtained from transgenic mice that have been engineered to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain locus are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy chain and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described, for example, by Green *et al.*, *Nature Genet.* 7:13 (1994), Lonberg *et al.*, *Nature* 368:856 (1994), and Taylor *et al.*, *Int. Immun.* 6:579 (1994).

Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography (see, for example, Coligan at pages 2.7.1-2.7.12 and pages 2.9.1-2.9.3; Baines *et al.*, "Purification of Immunoglobulin G (IgG)," in *Methods in Molecular Biology*, Vol. 10, pages 79-104 (The Humana Press, Inc. 1992)).

For particular uses, it may be desirable to prepare fragments of anti-Zsmf13 antibodies. Such antibody fragments can be obtained, for example, by proteolytic hydrolysis of the antibody. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. As an illustration, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin

to provide a 5S fragment denoted  $F(ab')_2$ . This fragment can be further cleaved using a thiol reducing agent to produce 3.5S Fab' monovalent fragments. Optionally, the cleavage reaction can be performed using a blocking group for the sulfhydryl groups that result from cleavage of disulfide linkages. As an alternative, an enzymatic cleavage using pepsin produces two monovalent Fab fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. patent No. 4,331,647, Nisonoff *et al.*, *Arch Biochem. Biophys.* 89:230 (1960), Porter, *Biochem. J.* 73:119 (1959), Edelman *et al.*, in *Methods in Enzymology Vol. 1*, page 422 (Academic Press 1967), and by Coligan at pages 2.8.1-2.8.10 and 2.10.-2.10.4.

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

For example, Fv fragments comprise an association of  $V_H$  and  $V_L$  chains. This association can be noncovalent, as described by Inbar *et al.*, *Proc. Nat'l Acad. Sci. USA* 69:2659 (1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde (see, for example, Sandhu, *Crit. Rev. Biotech.* 12:437 (1992)).

The Fv fragments may comprise  $V_H$  and  $V_L$  chains which are connected by a peptide linker. These single-chain antigen binding proteins (scFv) are prepared by constructing a structural gene comprising DNA sequences encoding the  $V_H$  and  $V_L$  domains which are connected by an oligonucleotide. The structural gene is inserted into an expression vector which is subsequently introduced into a host cell, such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing scFvs are described, for example, by Whitlow *et al.*, *Methods: A Companion to Methods in Enzymology* 2:97 (1991) (also see, Bird *et al.*, *Science* 242:423 (1988), Ladner *et al.*, U.S. Patent No. 4,946,778, Pack *et al.*, *Bio/Technology* 11:1271 (1993), and Sandhu, *supra*).

As an illustration, a scFV can be obtained by exposing lymphocytes to Zsmf13 polypeptide *in vitro*, and selecting antibody display libraries in phage or similar vectors (for instance, through use of immobilized or labeled Zsmf13 protein or peptide). Genes encoding polypeptides having potential Zsmf13 polypeptide binding domains can be obtained by screening random peptide libraries displayed on phage (phage display) or on bacteria, such as *E. coli*. Nucleotide sequences encoding the polypeptides can be obtained in a number of ways, such as through random mutagenesis and random polynucleotide synthesis. These random peptide display

libraries can be used to screen for peptides which interact with a known target which can be a protein or polypeptide, such as a ligand or receptor, a biological or synthetic macromolecule, or organic or inorganic substances. Techniques for creating and screening such random peptide display libraries are known in the art (Ladner *et al.*, U.S. Patent No. 5,223,409, Ladner *et al.*, U.S. Patent No. 4,946,778, Ladner *et al.*, U.S. Patent No. 5,403,484, Ladner *et al.*, U.S. Patent No. 5,571,698, and Kay *et al.*, *Phage Display of Peptides and Proteins* (Academic Press, Inc. 1996)) and random peptide display libraries and kits for screening such libraries are available commercially, for instance from CLONTECH Laboratories, Inc. (Palo Alto, CA), Invitrogen Inc. (San Diego, CA), New England Biolabs, Inc. (Beverly, MA), and Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Random peptide display libraries can be screened using the Zsmf13 sequences disclosed herein to identify proteins which bind to Zsmf13.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells (see, for example, Larrick *et al.*, *Methods: A Companion to Methods in Enzymology* 2:106 (1991), Courtenay-Luck, "Genetic Manipulation of Monoclonal Antibodies," in *Monoclonal Antibodies: Production, Engineering and Clinical Application*, Ritter *et al.* (eds.), page 166 (Cambridge University Press 1995), and Ward *et al.*, "Genetic Manipulation and Expression of Antibodies," in *Monoclonal Antibodies: Principles and Applications*, Birch *et al.*, (eds.), page 137 (Wiley-Liss, Inc. 1995)).

Alternatively, an anti-Zsmf13 antibody may be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementary determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain. Typical residues of human antibodies are then substituted in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by Orlandi *et al.*, *Proc. Nat'l Acad. Sci. USA* 86:3833 (1989). Techniques for producing humanized monoclonal antibodies are described, for example, by Jones *et al.*, *Nature* 321:522 (1986), Carter *et al.*, *Proc. Nat'l Acad. Sci. USA* 89:4285 (1992), Sandhu, *Crit. Rev.*

*Biotech.* 12:437 (1992), Singer *et al.*, *J. Immun.* 150:2844 (1993), Sudhir (ed.), *Antibody Engineering Protocols* (Humana Press, Inc. 1995), Kelley, "Engineering Therapeutic Antibodies," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 399-434 (John Wiley & Sons, Inc. 1996), and by Queen *et al.*, U.S. Patent No. 5,693,762 (1997).

Polyclonal anti-idiotypic antibodies can be prepared by immunizing animals with anti-Zsmf13 antibodies or antibody fragments, using standard techniques. See, for example, Green *et al.*, "Production of Polyclonal Antisera," in *Methods In Molecular Biology: Immunochemical Protocols*, Manson (ed.), pages 1-12 (Humana Press 1992). Also, see Coligan at pages 2.4.1-2.4.7. Alternatively, monoclonal anti-idiotypic antibodies can be prepared using anti-Zsmf13 antibodies or antibody fragments as immunogens with the techniques, described above. As another alternative, humanized anti-idiotypic antibodies or subhuman primate anti-idiotypic antibodies can be prepared using the above-described techniques. Methods for producing anti-idiotypic antibodies are described, for example, by Irie, U.S. Patent No. 5,208,146, Greene, *et al.*, U.S. Patent No. 5,637,677, and Varthakavi and Minocha, *J. Gen. Virol.* 77:1875 (1996).

#### **10. Detection of Zsmf13 Gene Expression and Examination of the Zsmf13 Chromosomal Locus**

Nucleic acid molecules can be used to detect the expression of a *Zsmf13* gene in a biological sample. Such probe molecules include double-stranded nucleic acid molecules comprising the nucleotide sequence of SEQ ID NO:1, or a fragment thereof, as well as single-stranded nucleic acid molecules having the complement of the nucleotide sequence of SEQ ID NO:1, or a fragment thereof. Probe molecules may be DNA, RNA, oligonucleotides, and the like.

Certain probes comprise a portion of the nucleotide sequence of nucleotides 225 to 821 of SEQ ID NO:1, or complement thereof. As used herein, the term "portion" refers to at least eight nucleotides to at least 20 or more nucleotides. Additional illustrative probes bind to certain functional domains of *Zsmf13*.

In a basic assay, a single-stranded probe molecule is incubated with RNA, isolated from a biological sample, under conditions of temperature and ionic strength that promote base pairing between the probe and target *Zsmf13* RNA species. After separating unbound probe from hybridized molecules, the amount of hybrids is detected.

Well-established hybridization methods of RNA detection include northern analysis and dot/slot blot hybridization (see, for example, Ausubel (1995) at pages 4-1 to 4-27, and Wu *et al.* (eds.), "Analysis of Gene Expression at the RNA Level," in *Methods in Gene Biotechnology*, pages 225-239 (CRC Press, Inc. 1997)).

5 Nucleic acid probes can be detectably labeled with radioisotopes such as  $^{32}\text{P}$  or  $^{35}\text{S}$ . Alternatively, Zsmf13 RNA can be detected with a nonradioactive hybridization method (see, for example, Isaac (ed.), *Protocols for Nucleic Acid Analysis by Nonradioactive Probes* (Humana Press, Inc. 1993)). Typically, nonradioactive detection is achieved by enzymatic conversion of chromogenic or chemiluminescent substrates. Illustrative

10 nonradioactive moieties include biotin, fluorescein, and digoxigenin.

Zsmf13 oligonucleotide probes are also useful for *in vivo* diagnosis. As an illustration,  $^{18}\text{F}$ -labeled oligonucleotides can be administered to a subject and visualized by positron emission tomography (Tavitian *et al.*, *Nature Medicine* 4:467 (1998)).

15 Numerous diagnostic procedures take advantage of the polymerase chain reaction (PCR) to increase sensitivity of detection methods. Standard techniques for performing PCR are well-known (see, generally, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), White (ed.), *PCR Protocols: Current Methods and Applications* (Humana Press, Inc. 1993), Cotter (ed.),

20 *Molecular Diagnosis of Cancer* (Humana Press, Inc. 1996), Hanausek and Walaszek (eds.), *Tumor Marker Protocols* (Humana Press, Inc. 1998), Lo (ed.), *Clinical Applications of PCR* (Humana Press, Inc. 1998), and Meltzer (ed.), *PCR in Bioanalysis* (Humana Press, Inc. 1998)).

One variation of PCR for diagnostic assays is reverse transcriptase-PCR (RT-PCR). In the RT-PCR technique, RNA is isolated from a biological sample,

25 reverse transcribed to cDNA, and the cDNA is incubated with Zsmf13 primers (see, for example, Wu *et al.* (eds.), "Rapid Isolation of Specific cDNAs or Genes by PCR," in *Methods in Gene Biotechnology*, pages 15-28 (CRC Press, Inc. 1997)). PCR is then performed and the products are analyzed using standard techniques.

30 As an illustration, RNA is isolated from biological sample using, for example, the guanidinium-thiocyanate cell lysis procedure described above. Alternatively, a solid-phase technique can be used to isolate mRNA from a cell lysate. A reverse transcription reaction can be primed with the isolated RNA using random oligonucleotides, short homopolymers of dT, or Zsmf13 anti-sense oligomers. Oligo-

35 dT primers offer the advantage that various mRNA nucleotide sequences are amplified that can provide control target sequences. Zsmf13 sequences are amplified by the

polymerase chain reaction using two flanking oligonucleotide primers that are typically 20 bases in length.

PCR amplification products can be detected using a variety of approaches. For example, PCR products can be fractionated by gel electrophoresis, and visualized by ethidium bromide staining. Alternatively, fractionated PCR products can be transferred to a membrane, hybridized with a detectably-labeled Zsmf13 probe, and examined by autoradiography. Additional alternative approaches include the use of digoxigenin-labeled deoxyribonucleic acid triphosphates to provide chemiluminescence detection, and the C-TRAK colorimetric assay.

Another approach for detection of Zsmf13 expression is cycling probe technology, in which a single-stranded DNA target binds with an excess of DNA-RNA-DNA chimeric probe to form a complex, the RNA portion is cleaved with RNAase H, and the presence of cleaved chimeric probe is detected (see, for example, Beggs *et al.*, *J. Clin. Microbiol.* 34:2985 (1996), Bekkaoui *et al.*, *Biotechniques* 20:240 (1996)). Alternative methods for detection of Zsmf13 sequences can utilize approaches such as nucleic acid sequence-based amplification, cooperative amplification of templates by cross-hybridization, and the ligase chain reaction (see, for example, Marshall *et al.*, U.S. Patent No. 5,686,272 (1997), Dyer *et al.*, *J. Virol. Methods* 60:161 (1996), Ehricht *et al.*, *Eur. J. Biochem.* 243:358 (1997), and Chadwick *et al.*, *J. Virol. Methods* 70:59 (1998)). Other standard methods are known to those of skill in the art.

Zsmf13 probes and primers can also be used to detect and to localize Zsmf13 gene expression in tissue samples. Methods for such *in situ* hybridization are well-known to those of skill in the art (see, for example, Choo (ed.), *In Situ Hybridization Protocols* (Humana Press, Inc. 1994), Wu *et al.* (eds.), "Analysis of Cellular DNA or Abundance of mRNA by Radioactive *In Situ* Hybridization (RISH)," in *Methods in Gene Biotechnology*, pages 259-278 (CRC Press, Inc. 1997), and Wu *et al.* (eds.), "Localization of DNA or Abundance of mRNA by Fluorescence *In Situ* Hybridization (RISH)," in *Methods in Gene Biotechnology*, pages 279-289 (CRC Press, Inc. 1997)). Various additional diagnostic approaches are well-known to those of skill in the art (see, for example, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), Coleman and Tsongalis, *Molecular Diagnostics* (Humana Press, Inc. 1996), and Elles, *Molecular Diagnosis of Genetic Diseases* (Humana Press, Inc., 1996)).

Nucleic acid molecules comprising Zsmf13 nucleotide sequences can be used to determine whether a subject's chromosomes contain a mutation in the

*Zsmf13* gene. Detectable chromosomal aberrations at the *Zsmf13* gene locus include, but are not limited to, aneuploidy, gene copy number changes, insertions, deletions, restriction site changes and rearrangements. Of particular interest are genetic alterations that inactivate the *Zsmf13* gene.

5 Aberrations associated with the *Zsmf13* locus can be detected using nucleic acid molecules of the present invention by employing molecular genetic techniques, such as restriction fragment length polymorphism analysis, short tandem repeat analysis employing PCR techniques, amplification-refractory mutation system analysis, single-strand conformation polymorphism detection, RNase cleavage  
10 methods, denaturing gradient gel electrophoresis, fluorescence-assisted mismatch analysis, and other genetic analysis techniques known in the art (see, for example, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), Marian, *Chest* 108:255 (1995), Coleman and Tsongalis, *Molecular Diagnostics* (Human Press, Inc. 1996), Elles (ed.) *Molecular Diagnosis of Genetic Diseases* (Humana Press, Inc. 1996), Landegren (ed.), *Laboratory Protocols for Mutation Detection* (Oxford University Press 1996), Birren *et al.* (eds.), *Genome Analysis, Vol. 2: Detecting Genes* (Cold Spring Harbor Laboratory Press 1998), Dracopoli *et al.* (eds.), *Current Protocols in Human Genetics* (John Wiley & Sons 1998), and Richards and Ward, "Molecular Diagnostic Testing," in *Principles of Molecular Medicine*,  
15 20 pages 83-88 (Humana Press, Inc. 1998)).

The chromosomal location of the *Zsmf13* gene can be determined using radiation hybrid mapping, which is a somatic cell genetic technique developed for constructing high-resolution, contiguous maps of mammalian chromosomes (Cox *et al.*, *Science* 250:245 (1990)). Partial or full knowledge of a gene's sequence allows  
25 one to design PCR primers suitable for use with chromosomal radiation hybrid mapping panels. Radiation hybrid mapping panels are commercially available which cover the entire human genome, such as the Stanford G3 RH Panel and the GeneBridge 4 RH Panel (Research Genetics, Inc., Huntsville, AL). These panels enable rapid, PCR-based chromosomal localizations and ordering of genes, sequence-  
30 tagged sites, and other nonpolymorphic and polymorphic markers within a region of interest. This includes establishing directly proportional physical distances between newly discovered genes of interest and previously mapped markers.

The protein truncation test is also useful for detecting the inactivation of a gene in which translation-terminating mutations produce only portions of the  
35 encoded protein (see, for example, Stoppa-Lyonnet *et al.*, *Blood* 91:3920 (1998)). According to this approach, RNA is isolated from a biological sample, and used to

synthesize cDNA. PCR is then used to amplify the *Zsmf13* target sequence and to introduce an RNA polymerase promoter, a translation initiation sequence, and an in-frame ATG triplet. PCR products are transcribed using an RNA polymerase, and the transcripts are translated *in vitro* with a T7-coupled reticulocyte lysate system. The translation products are then fractionated by SDS-PAGE to determine the lengths of the translation products. The protein truncation test is described, for example, by Dracopoli *et al.* (eds.), *Current Protocols in Human Genetics*, pages 9.11.1 - 9.11.18 (John Wiley & Sons 1998).

The present invention also contemplates kits for performing an assay for *Zsmf13* gene expression, or to examine the *Zsmf13* locus. Such kits comprise nucleic acid probes, such as double-stranded nucleic acid molecules comprising the nucleotide sequence of SEQ ID NO:1, or a fragment thereof, as well as single-stranded nucleic acid molecules having the complement of the nucleotide sequence of SEQ ID NO:1, or a fragment thereof. Probe molecules may be DNA, RNA, oligonucleotides, and the like. Kits may comprise nucleic acid primers for performing PCR.

Such a kit can contain all the necessary elements to perform a nucleic acid diagnostic assay described above. A kit will comprise at least one container comprising a *Zsmf13* probe or primer. The kit may also comprise a second container comprising one or more reagents capable of indicating the presence of *Zsmf13* sequences. Examples of such indicator reagents include detectable labels such as radioactive labels, fluorochromes, chemiluminescent agents, and the like. A kit may also comprise a means for conveying to the user that the *Zsmf13* probes and primers are used to detect *Zsmf13* gene expression. For example, written instructions may state that the enclosed nucleic acid molecules can be used to detect either a nucleic acid molecule that encodes *Zsmf13*, or a nucleic acid molecule having a nucleotide sequence that is complementary to a *Zsmf13*-encoding nucleotide sequence. The written material can be applied directly to a container, or the written material can be provided in the form of a packaging insert.

## **11. Detection of *Zsmf13* Protein with Anti-*Zsmf13* Antibodies**

The present invention contemplates the use of anti-*Zsmf13* antibodies to screen biological samples *in vitro* for the presence of *Zsmf13*. In one type of *in vitro* assay, anti-*Zsmf13* antibodies are used in liquid phase. For example, the presence of *Zsmf13* in a biological sample can be tested by mixing the biological sample with a trace amount of labeled *Zsmf13* and an anti-*Zsmf13* antibody under conditions that promote binding between *Zsmf13* and its antibody. Complexes of *Zsmf13* and anti-*Zsmf13* in



the sample can be separated from the reaction mixture by contacting the complex with an immobilized protein which binds with the antibody, such as an Fc antibody or *Staphylococcus* protein A. The concentration of Zsmf13 in the biological sample will be inversely proportional to the amount of labeled Zsmf13 bound to the antibody and directly related to the amount of free labeled Zsmf13.

Alternatively, *in vitro* assays can be performed in which anti-Zsmf13 antibody is bound to a solid-phase carrier. For example, antibody can be attached to a polymer, such as aminodextran, in order to link the antibody to an insoluble support such as a polymer-coated bead, a plate or a tube. Other suitable *in vitro* assays will be readily apparent to those of skill in the art.

In another approach, anti-Zsmf13 antibodies can be used to detect Zsmf13 in tissue sections prepared from a biopsy specimen. Such immunochemical detection can be used to determine the relative abundance of Zsmf13 and to determine the distribution of Zsmf13 in the examined tissue. General immunochemistry techniques are well established (see, for example, Ponder, "Cell Marking Techniques and Their Application," in *Mammalian Development: A Practical Approach*, Monk (ed.), pages 115-38 (IRL Press 1987), Coligan at pages 5.8.1-5.8.8, Ausubel (1995) at pages 14.6.1 to 14.6.13 (Wiley Interscience 1990), and Manson (ed.), *Methods In Molecular Biology, Vol.10: Immunochemical Protocols* (The Humana Press, Inc. 1992)).

Immunochemical detection can be performed by contacting a biological sample with an anti-Zsmf13 antibody, and then contacting the biological sample with a detectably labeled molecule which binds to the antibody. For example, the detectably labeled molecule can comprise an antibody moiety that binds to anti-Zsmf13 antibody. Alternatively, the anti-Zsmf13 antibody can be conjugated with avidin/streptavidin (or biotin) and the detectably labeled molecule can comprise biotin (or avidin/streptavidin). Numerous variations of this basic technique are well-known to those of skill in the art.

Alternatively, an anti-Zsmf13 antibody can be conjugated with a detectable label to form an anti-Zsmf13 immunoconjugate. Suitable detectable labels include, for example, a radioisotope, a fluorescent label, a chemiluminescent label, an enzyme label, a bioluminescent label or colloidal gold. Methods of making and detecting such detectably-labeled immunoconjugates are well-known to those of ordinary skill in the art, and are described in more detail below.

The detectable label can be a radioisotope that is detected by autoradiography. Isotopes that are particularly useful for the purpose of the present invention are  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$  and  $^{14}\text{C}$ .

Anti-Zsmf13 immunoconjugates can also be labeled with a fluorescent compound. The presence of a fluorescently-labeled antibody is determined by exposing the immunoconjugate to light of the proper wavelength and detecting the resultant fluorescence. Fluorescent labeling compounds include fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

Alternatively, anti-Zsmf13 immunoconjugates can be detectably labeled by coupling an antibody component to a chemiluminescent compound. The presence of the chemiluminescent-tagged immunoconjugate is determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of chemiluminescent labeling compounds include luminol, isoluminol, an aromatic acridinium ester, an imidazole, an acridinium salt and an oxalate ester.

Similarly, a bioluminescent compound can be used to label anti-Zsmf13 immunoconjugates of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Bioluminescent compounds that are useful for labeling include luciferin, luciferase and aequorin.

Alternatively, anti-Zsmf13 immunoconjugates can be detectably labeled by linking an anti-Zsmf13 antibody component to an enzyme. When the anti-Zsmf13-enzyme conjugate is incubated in the presence of the appropriate substrate, the enzyme moiety reacts with the substrate to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or visual means. Examples of enzymes that can be used to detectably label polyspecific immunoconjugates include  $\beta$ -galactosidase, glucose oxidase, peroxidase and alkaline phosphatase.

Those of skill in the art will know of other suitable labels which can be employed in accordance with the present invention. The binding of marker moieties to anti-Zsmf13 antibodies can be accomplished using standard techniques known to the art. Typical methodology in this regard is described by Kennedy *et al.*, *Clin. Chim. Acta* 70:1 (1976), Schurs *et al.*, *Clin. Chim. Acta* 81:1 (1977), Shih *et al.*, *Int'l J. Cancer* 46:1101 (1990), Stein *et al.*, *Cancer Res.* 50:1330 (1990), and Coligan, *supra*.

Moreover, the convenience and versatility of immunochemical detection can be enhanced by using anti-Zsmf13 antibodies that have been conjugated with avidin, streptavidin, and biotin (see, for example, Wilchek *et al.* (eds.), "Avidin-Biotin Technology," *Methods In Enzymology*, Vol. 184 (Academic Press 1990), and Bayer *et al.*, "Immunochemical Applications of Avidin-Biotin Technology," in *Methods In*

*Molecular Biology, Vol. 10*, Manson (ed.), pages 149-162 (The Humana Press, Inc. 1992).

Methods for performing immunoassays are well-established. See, for example, Cook and Self, "Monoclonal Antibodies in Diagnostic Immunoassays," in  
5 *Monoclonal Antibodies: Production, Engineering, and Clinical Application*, Ritter and Ladyman (eds.), pages 180-208, (Cambridge University Press, 1995), Perry, "The Role of Monoclonal Antibodies in the Advancement of Immunoassay Technology," in  
*Monoclonal Antibodies: Principles and Applications*, Birch and Lennox (eds.), pages 107-120 (Wiley-Liss, Inc. 1995), and Diamandis, *Immunoassay* (Academic Press, Inc.  
10 1996).

In a related approach, biotin- or FITC-labeled Zsmf13 can be used to identify cells that bind Zsmf13. Such can binding can be detected, for example, using flow cytometry.

The present invention also contemplates kits for performing an  
15 immunological diagnostic assay for *Zsmf13* gene expression. Such kits comprise at least one container comprising an anti-Zsmf13 antibody, or antibody fragment. A kit may also comprise a second container comprising one or more reagents capable of indicating the presence of Zsmf13 antibody or antibody fragments. Examples of such  
20 indicator reagents include detectable labels such as a radioactive label, a fluorescent label, a chemiluminescent label, an enzyme label, a bioluminescent label, colloidal gold, and the like. A kit may also comprise a means for conveying to the user that Zsmf13 antibodies or antibody fragments are used to detect Zsmf13 protein. For example, written instructions may state that the enclosed antibody or antibody fragment can be used to detect Zsmf13. The written material can be applied directly to a container, or  
25 the written material can be provided in the form of a packaging insert.

## **12. Therapeutic Uses of Zsmf13 Polypeptides**

The present invention includes the use of proteins, polypeptides, and peptides having Zsmf13 activity (such as Zsmf13 polypeptides, Zsmf13 analogs,  
30 active Zsmf13 anti-idiotypic antibodies, and Zsmf13 fusion proteins) to a subject who lacks an adequate amount of this polypeptide. For example, these molecules may be useful in the regeneration and direction of neurite outgrowths following stroke, and neurodegenerative disease. In contrast, Zsmf13 antagonists (e.g., anti-Zsmf13 antibodies or anti-Zsmf13 anti-idiotypic antibodies that are biologically inactive) can  
35 be used to treat a subject who produces an excess of Zsmf13.

Generally, the dosage of administered polypeptide, protein or peptide will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition and previous medical history. Typically, it is desirable to provide the recipient with a dosage of a molecule having Zsmf13 activity, which is in the range of from about 1 pg/kg to 10 mg/kg (amount of agent/body weight of patient), although a lower or higher dosage also may be administered as circumstances dictate.

Administration of a molecule having Zsmf13 activity to a subject can be intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, intrapleural, intrathecal, by perfusion through a regional catheter, or by direct intralesional injection. When administering therapeutic proteins by injection, the administration may be by continuous infusion or by single or multiple boluses.

A pharmaceutical composition comprising a protein, polypeptide, or peptide having Zsmf13 activity can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the therapeutic proteins are combined in a mixture with a pharmaceutically acceptable carrier. A composition is said to be a "pharmaceutically acceptable carrier" if its administration can be tolerated by a recipient patient. Sterile phosphate-buffered saline is one example of a pharmaceutically acceptable carrier. Other suitable carriers are well-known to those in the art. See, for example, Gennaro (ed.), *Remington's Pharmaceutical Sciences*, 19th Edition (Mack Publishing Company 1995).

For purposes of therapy, molecules having Zsmf13 activity and a pharmaceutically acceptable carrier are administered to a patient in a therapeutically effective amount. A combination of a protein, polypeptide, or peptide having Zsmf13 activity and a pharmaceutically acceptable carrier is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient.

A pharmaceutical composition comprising molecules having Zsmf13 activity can be furnished in liquid form, or in solid form. Liquid forms, including liposome-encapsulated formulations, are illustrated by injectable solutions and oral suspensions. Exemplary solid forms include capsules, tablets, and controlled-release forms, such as a miniosmotic pump or an implant. Other dosage forms can be devised by those skilled in the art, as shown, for example, by Ansel and Popovich, *Pharmaceutical Dosage Forms and Drug Delivery Systems*, 5<sup>th</sup> Edition (Lea & Febiger 1990), Gennaro (ed.), *Remington's Pharmaceutical Sciences*, 19<sup>th</sup> Edition

(Mack Publishing Company 1995), and by Ranade and Hollinger, *Drug Delivery Systems* (CRC Press 1996).

As an illustration, Zsmf13 pharmaceutical compositions may be supplied as a kit comprising a container that comprises Zsmf13. Zsmf13 can be provided in the form of an injectable solution for single or multiple doses, or as a sterile powder that will be reconstituted before injection. Such a kit may further comprise written information on indications and usage of the pharmaceutical composition. Moreover, such information may include a statement that the Zsmf13 composition is contraindicated in patients with known hypersensitivity to Zsmf13.

### 13. Therapeutic Uses of Zsmf13 Nucleotide Sequences

The present invention includes the use of Zsmf13 nucleotide sequences to provide Zsmf13 polypeptides to a subject in need of such treatment. In addition, a therapeutic expression vector can be provided that inhibits *Zsmf13* gene expression, such as an anti-sense molecule, a ribozyme, or an external guide sequence molecule.

There are numerous approaches to introduce a *Zsmf13* gene to a subject, including the use of recombinant host cells that express Zsmf13, delivery of naked nucleic acid encoding Zsmf13, use of a cationic lipid carrier with a nucleic acid molecule that encodes Zsmf13, and the use of viruses that express Zsmf13, such as recombinant retroviruses, recombinant adeno-associated viruses, recombinant adenoviruses, and recombinant Herpes simplex viruses [HSV] (see, for example, Mulligan, *Science* 260:926 (1993), Rosenberg *et al.*, *Science* 242:1575 (1988), LaSalle *et al.*, *Science* 259:988 (1993), Wolff *et al.*, *Science* 247:1465 (1990), Breakfield and Deluca, *The New Biologist* 3:203 (1991)). In an *ex vivo* approach, for example, cells are isolated from a subject, transfected with a vector that expresses a *Zsmf13* gene, and then transplanted into the subject.

In order to effect expression of a *Zsmf13* gene, an expression vector is constructed in which a nucleotide sequence encoding a *Zsmf13* gene is operably linked to a core promoter, and optionally a regulatory element, to control gene transcription. The general requirements of an expression vector are described above.

Alternatively, a *Zsmf13* gene can be delivered using recombinant viral vectors, including for example, adenoviral vectors (*e.g.*, Kass-Eisler *et al.*, *Proc. Nat'l Acad. Sci. USA* 90:11498 (1993), Kolls *et al.*, *Proc. Nat'l Acad. Sci. USA* 91:215 (1994), Li *et al.*, *Hum. Gene Ther.* 4:403 (1993), Vincent *et al.*, *Nat. Genet.* 5:130 (1993), and Zabner *et al.*, *Cell* 75:207 (1993)), adenovirus-associated viral vectors (Flotte *et al.*, *Proc. Nat'l Acad. Sci. USA* 90:10613 (1993)), alphaviruses such as

Semliki Forest Virus and Sindbis Virus (Hertz and Huang, *J. Vir.* 66:857 (1992), Raju and Huang, *J. Vir.* 65:2501 (1991), and Xiong *et al.*, *Science* 243:1188 (1989)), herpes viral vectors (*e.g.*, U.S. Patent Nos. 4,769,331, 4,859,587, 5,288,641 and 5,328,688), parvovirus vectors (Koering *et al.*, *Hum. Gene Therap.* 5:457 (1994)), pox virus  
5 vectors (Ozaki *et al.*, *Biochem. Biophys. Res. Comm.* 193:653 (1993), Panicali and Paoletti, *Proc. Nat'l Acad. Sci. USA* 79:4927 (1982)), pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch *et al.*, *Proc. Nat'l Acad. Sci. USA* 86:317 (1989), and Flexner *et al.*, *Ann. N.Y. Acad. Sci.* 569:86 (1989)), and retroviruses (*e.g.*, Baba *et al.*, *J. Neurosurg* 79:729 (1993), Ram *et al.*, *Cancer Res.* 53:83 (1993), Takamiya *et al.*,  
10 *J. Neurosci. Res* 33:493 (1992), Vile and Hart, *Cancer Res.* 53:962 (1993), Vile and Hart, *Cancer Res.* 53:3860 (1993), and Anderson *et al.*, U.S. Patent No. 5,399,346). Within various embodiments, either the viral vector itself, or a viral particle which contains the viral vector may be utilized in the methods and compositions described below.

15 As an illustration of one system, adenovirus, a double-stranded DNA virus, is a well-characterized gene transfer vector for delivery of a heterologous nucleic acid molecule (for a review, see Becker *et al.*, *Meth. Cell Biol.* 43:161 (1994); Douglas and Curiel, *Science & Medicine* 4:44 (1997)). The adenovirus system offers several advantages including: (i) the ability to accommodate relatively large DNA  
20 inserts, (ii) the ability to be grown to high-titer, (iii) the ability to infect a broad range of mammalian cell types, and (iv) the ability to be used with many different promoters including ubiquitous, tissue specific, and regulatable promoters. In addition, adenoviruses can be administered by intravenous injection, because the viruses are stable in the bloodstream.

25 Using adenovirus vectors where portions of the adenovirus genome are deleted, inserts are incorporated into the viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. In an exemplary system, the essential E1 gene is deleted from the viral vector, and the virus will not replicate unless the E1 gene is provided by the host cell. When intravenously administered to  
30 intact animals, adenovirus primarily targets the liver. Although an adenoviral delivery system with an E1 gene deletion cannot replicate in the host cells, the host's tissue will express and process an encoded heterologous protein. Host cells will also secrete the heterologous protein if the corresponding gene includes a secretory signal sequence. Secreted proteins will enter the circulation from tissue that expresses the heterologous  
35 gene (*e.g.*, the highly vascularized liver).

Moreover, adenoviral vectors containing various deletions of viral genes can be used to reduce or eliminate immune responses to the vector. Such adenoviruses are E1-deleted, and in addition, contain deletions of E2A or E4 (Lusky *et al.*, *J. Virol.* 72:2022 (1998); Raper *et al.*, *Human Gene Therapy* 9:671 (1998)). The deletion of E2b has also been reported to reduce immune responses (Amalfitano *et al.*, *J. Virol.* 72:926 (1998)). By deleting the entire adenovirus genome, very large inserts of heterologous DNA can be accommodated. Generation of so called "gutless" adenoviruses, where all viral genes are deleted, are particularly advantageous for insertion of large inserts of heterologous DNA (for a review, see Yeh. and Perricaudet, *FASEB J.* 11:615 (1997)).

High titer stocks of recombinant viruses capable of expressing a therapeutic gene can be obtained from infected mammalian cells using standard methods. For example, recombinant HSV can be prepared in Vero cells, as described by Brandt *et al.*, *J. Gen. Virol.* 72:2043 (1991), Herold *et al.*, *J. Gen. Virol.* 75:1211 (1994), Visalli and Brandt, *Virology* 185:419 (1991), Grau *et al.*, *Invest. Ophthalmol. Vis. Sci.* 30:2474 (1989), Brandt *et al.*, *J. Virol. Meth.* 36:209 (1992), and by Brown and MacLean (eds.), *HSV Virus Protocols* (Humana Press 1997).

Alternatively, an expression vector comprising a *Zsmf13* gene can be introduced into a subject's cells by lipofection *in vivo* using liposomes. Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker (Felgner *et al.*, *Proc. Nat'l Acad. Sci. USA* 84:7413 (1987); Mackey *et al.*, *Proc. Nat'l Acad. Sci. USA* 85:8027 (1988)). The use of lipofection to introduce exogenous genes into specific organs *in vivo* has certain practical advantages. Liposomes can be used to direct transfection to particular cell types, which is particularly advantageous in a tissue with cellular heterogeneity, such as the pancreas, liver, kidney, and brain. Lipids may be chemically coupled to other molecules for the purpose of targeting. Targeted peptides (*e.g.*, hormones or neurotransmitters), proteins such as antibodies, or non-peptide molecules can be coupled to liposomes chemically.

Electroporation is another alternative mode of administration of a *Zsmf13* nucleic acid molecules. For example, Aihara and Miyazaki, *Nature Biotechnology* 16:867 (1998), have demonstrated the use of *in vivo* electroporation for gene transfer into muscle.

In an alternative approach to gene therapy, a therapeutic gene may encode a *Zsmf13* anti-sense RNA that inhibits the expression of *Zsmf13*. Suitable

sequences for Zsmf13 anti-sense molecules can be derived from the nucleotide sequences of Zsmf13 disclosed herein.

Alternatively, an expression vector can be constructed in which a regulatory element is operably linked to a nucleotide sequence that encodes a ribozyme. Ribozymes can be designed to express endonuclease activity that is directed to a certain target sequence in a mRNA molecule (see, for example, Draper and Macejak, U.S. Patent No. 5,496,698, McSwiggen, U.S. Patent No. 5,525,468, Chowrira and McSwiggen, U.S. Patent No. 5,631,359, and Robertson and Goldberg, U.S. Patent No. 5,225,337). In the context of the present invention, ribozymes include nucleotide sequences that bind with Zsmf13 mRNA.

In another approach, expression vectors can be constructed in which a regulatory element directs the production of RNA transcripts capable of promoting RNase P-mediated cleavage of mRNA molecules that encode a *Zsmf13* gene. According to this approach, an external guide sequence can be constructed for directing the endogenous ribozyme, RNase P, to a particular species of intracellular mRNA, which is subsequently cleaved by the cellular ribozyme (see, for example, Altman *et al.*, U.S. Patent No. 5,168,053, Yuan *et al.*, *Science* 263:1269 (1994), Pace *et al.*, international publication No. WO 96/18733, George *et al.*, international publication No. WO 96/21731, and Werner *et al.*, international publication No. WO 97/33991). Preferably, the external guide sequence comprises a ten to fifteen nucleotide sequence complementary to *Zsmf13* mRNA, and a 3'-NCCA nucleotide sequence, wherein N is preferably a purine. The external guide sequence transcripts bind to the targeted mRNA species by the formation of base pairs between the mRNA and the complementary external guide sequences, thus promoting cleavage of mRNA by RNase P at the nucleotide located at the 5'-side of the base-paired region.

In general, the dosage of a composition comprising a therapeutic vector having a Zsmf13 nucleotide acid sequence, such as a recombinant virus, will vary depending upon such factors as the subject's age, weight, height, sex, general medical condition and previous medical history. Suitable routes of administration of therapeutic vectors include intravenous injection, intraarterial injection, intraperitoneal injection, intramuscular injection, intratumoral injection, and injection into a cavity that contains a tumor.

A composition comprising viral vectors, non-viral vectors, or a combination of viral and non-viral vectors of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby vectors or viruses are combined in a mixture with a pharmaceutically



acceptable carrier. As noted above, a composition, such as phosphate-buffered saline is said to be a "pharmaceutically acceptable carrier" if its administration can be tolerated by a recipient subject. Other suitable carriers are well-known to those in the art (see, for example, *Remington's Pharmaceutical Sciences*, 19th Ed. (Mack Publishing Co. 1995), and *Gilman's the Pharmacological Basis of Therapeutics*, 7th Ed. (MacMillan Publishing Co. 1985)).

For purposes of therapy, a therapeutic gene expression vector, or a recombinant virus comprising such a vector, and a pharmaceutically acceptable carrier are administered to a subject in a therapeutically effective amount. A combination of an expression vector (or virus) and a pharmaceutically acceptable carrier is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient subject.

When the subject treated with a therapeutic gene expression vector or a recombinant virus is a human, then the therapy is preferably somatic cell gene therapy. That is, the preferred treatment of a human with a therapeutic gene expression vector or a recombinant virus does not entail introducing into cells a nucleic acid molecule that can form part of a human germ line and be passed onto successive generations (i.e., human germ line gene therapy).

#### 14. Production of Transgenic Mice

Transgenic mice can be engineered to over-express the *Zsmf13* gene in all tissues or under the control of a tissue-specific or tissue-preferred regulatory element. These over-producers of *Zsmf13* can be used to characterize the phenotype that results from over-expression, and the transgenic animals can serve as models for human disease caused by excess *Zsmf13*. Transgenic mice that over-express *Zsmf13* also provide model bioreactors for production of *Zsmf13* in the milk or blood of larger animals. Methods for producing transgenic mice are well-known to those of skill in the art (see, for example, Jacob, "Expression and Knockout of Interferons in Transgenic Mice," in *Overexpression and Knockout of Cytokines in Transgenic Mice*, Jacob (ed.), pages 111-124 (Academic Press, Ltd. 1994), Monastersky and Robl (eds.), *Strategies in Transgenic Animal Science* (ASM Press 1995), and Abbud and Nilson, "Recombinant Protein Expression in Transgenic Mice," in *Gene Expression Systems: Using Nature for the Art of Expression*, Fernandez and Hoeffler (eds.), pages 367-397 (Academic Press, Inc. 1999)).

For example, a method for producing a transgenic mouse that expresses a *Zsmf13* gene can begin with adult, fertile males (studs) (B6C3f1, 2-8 months of age (Taconic Farms, Germantown, NY)), vasectomized males (duds) (B6D2f1, 2-8 months, (Taconic Farms)), prepubescent fertile females (donors) (B6C3f1, 4-5 weeks, (Taconic Farms)) and adult fertile females (recipients) (B6D2f1, 2-4 months, (Taconic Farms)). The donors are acclimated for one week and then injected with approximately 8 IU/mouse of Pregnant Mare's Serum gonadotrophin (Sigma Chemical Company; St. Louis, MO) I.P., and 46-47 hours later, 8 IU/mouse of human Chorionic Gonadotropin (hCG (Sigma)) I.P. to induce superovulation. Donors are mated with studs subsequent to hormone injections. Ovulation generally occurs within 13 hours of hCG injection. Copulation is confirmed by the presence of a vaginal plug the morning following mating.

Fertilized eggs are collected under a surgical scope. The oviducts are collected and eggs are released into urinanalysis slides containing hyaluronidase (Sigma). Eggs are washed once in hyaluronidase, and twice in Whitten's W640 medium (described, for example, by Menino and O'Claray, *Biol. Reprod.* 77:159 (1986), and Dienhart and Downs, *Zygote* 4:129 (1996)) that has been incubated with 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub> at 37°C. The eggs are then stored in a 37°C/5% CO<sub>2</sub> incubator until microinjection.

Ten to twenty micrograms of plasmid DNA containing a *Zsmf13* encoding sequence is linearized, gel-purified, and resuspended in 10 mM Tris-HCl (pH 7.4), 0.25 mM EDTA (pH 8.0), at a final concentration of 5-10 nanograms per microliter for microinjection. For example, the *Zsmf13* encoding sequences can encode a polypeptide comprising amino acid residues 20 to 218 of SEQ ID NO:2.

Plasmid DNA is microinjected into harvested eggs contained in a drop of W640 medium overlaid by warm, CO<sub>2</sub>-equilibrated mineral oil. The DNA is drawn into an injection needle (pulled from a 0.75mm ID, 1mm OD borosilicate glass capillary), and injected into individual eggs. Each egg is penetrated with the injection needle, into one or both of the haploid pronuclei.

Picoliters of DNA are injected into the pronuclei, and the injection needle withdrawn without coming into contact with the nucleoli. The procedure is repeated until all the eggs are injected. Successfully microinjected eggs are transferred into an organ tissue-culture dish with pre-gassed W640 medium for storage overnight in a 37°C/5% CO<sub>2</sub> incubator.

The following day, two-cell embryos are transferred into pseudopregnant recipients. The recipients are identified by the presence of copulation

plugs, after copulating with vasectomized duds. Recipients are anesthetized and shaved on the dorsal left side and transferred to a surgical microscope. A small incision is made in the skin and through the muscle wall in the middle of the abdominal area outlined by the ribcage, the saddle, and the hind leg, midway between  
5 knee and spleen. The reproductive organs are exteriorized onto a small surgical drape. The fat pad is stretched out over the surgical drape, and a baby serrefine (Roboz, Rockville, MD) is attached to the fat pad and left hanging over the back of the mouse, preventing the organs from sliding back in.

With a fine transfer pipette containing mineral oil followed by  
10 alternating W640 and air bubbles, 12-17 healthy two-cell embryos from the previous day's injection are transferred into the recipient. The swollen ampulla is located and holding the oviduct between the ampulla and the bursa, a nick in the oviduct is made with a 28 g needle close to the bursa, making sure not to tear the ampulla or the bursa.

The pipette is transferred into the nick in the oviduct, and the embryos  
15 are blown in, allowing the first air bubble to escape the pipette. The fat pad is gently pushed into the peritoneum, and the reproductive organs allowed to slide in. The peritoneal wall is closed with one suture and the skin closed with a wound clip. The mice recuperate on a 37°C slide warmer for a minimum of four hours.

The recipients are returned to cages in pairs, and allowed 19-21 days  
20 gestation. After birth, 19-21 days postpartum is allowed before weaning. The weanlings are sexed and placed into separate sex cages, and a 0.5 cm biopsy (used for genotyping) is snipped off the tail with clean scissors.

Genomic DNA is prepared from the tail snips using, for example, a  
QIAGEN DNEASY kit following the manufacturer's instructions. Genomic DNA is  
25 analyzed by PCR using primers designed to amplify a *Zsmf13* gene or a selectable marker gene that was introduced in the same plasmid. After animals are confirmed to be transgenic, they are back-crossed into an inbred strain by placing a transgenic female with a wild-type male, or a transgenic male with one or two wild-type female(s). As pups are born and weaned, the sexes are separated, and their tails  
30 snipped for genotyping.

To check for expression of a transgene in a live animal, a partial  
hepatectomy is performed. A surgical prep is made of the upper abdomen directly below the zyphoid process. Using sterile technique, a small 1.5-2 cm incision is made below the sternum and the left lateral lobe of the liver exteriorized. Using 4-0 silk, a  
35 tie is made around the lower lobe securing it outside the body cavity. An atraumatic clamp is used to hold the tie while a second loop of absorbable Dexon (American

Cyanamid; Wayne, N.J.) is placed proximal to the first tie. A distal cut is made from the Dexon tie and approximately 100 mg of the excised liver tissue is placed in a sterile petri dish. The excised liver section is transferred to a 14 ml polypropylene round bottom tube and snap frozen in liquid nitrogen and then stored on dry ice. The surgical site is closed with suture and wound clips, and the animal's cage placed on a 37°C heating pad for 24 hours post operatively. The animal is checked daily post operatively and the wound clips removed 7-10 days after surgery. The expression level of *Zsmf13* mRNA is examined for each transgenic mouse using an RNA solution hybridization assay or polymerase chain reaction.

In addition to producing transgenic mice that over-express *Zsmf13*, it is useful to engineer transgenic mice with either abnormally low or no expression of the gene. Such transgenic mice provide useful models for diseases associated with a lack of *Zsmf13*. As discussed above, *Zsmf13* gene expression can be inhibited using anti-sense genes, ribozyme genes, or external guide sequence genes. To produce transgenic mice that under-express the *Zsmf13* gene, such inhibitory sequences are targeted to *Zsmf13* mRNA. Methods for producing transgenic mice that have abnormally low expression of a particular gene are known to those in the art (see, for example, Wu *et al.*, "Gene Underexpression in Cultured Cells and Animals by Antisense DNA and RNA Strategies," in *Methods in Gene Biotechnology*, pages 205-224 (CRC Press 1997)).

An alternative approach to producing transgenic mice that have little or no *Zsmf13* gene expression is to generate mice having at least one normal *Zsmf13* allele replaced by a nonfunctional *Zsmf13* gene. One method of designing a nonfunctional *Zsmf13* gene is to insert another gene, such as a selectable marker gene, within a nucleic acid molecule that encodes *Zsmf13*. Standard methods for producing these so-called "knockout mice" are known to those skilled in the art (see, for example, Jacob, "Expression and Knockout of Interferons in Transgenic Mice," in *Overexpression and Knockout of Cytokines in Transgenic Mice*, Jacob (ed.), pages 111-124 (Academic Press, Ltd. 1994), and Wu *et al.*, "New Strategies for Gene Knockout," in *Methods in Gene Biotechnology*, pages 339-365 (CRC Press 1997)).

## CLAIMS

What is claimed is:

1. An isolated polypeptide, comprising an amino acid sequence that is at least 70% identical to amino acid residues 20 to 218 of SEQ ID NO:2, wherein the isolated polypeptide specifically binds with an antibody that specifically binds with a polypeptide consisting of the amino acid sequence of SEQ ID NO:2.
2. The isolated polypeptide of claim 1, wherein the isolated polypeptide has an amino acid sequence that is at least 80% identical to amino acid residues 20 to 218 of SEQ ID NO:2.
3. The isolated polypeptide of claim 1, wherein the isolated polypeptide has an amino acid sequence that is at least 90% identical to amino acid residues 20 to 218 of SEQ ID NO:2.
4. The isolated polypeptide of claim 1, comprising an amino acid sequence consisting of amino acid residues 20 to 218 of SEQ ID NO:2.
5. The isolated polypeptide of claim 4, wherein the polypeptide comprises an amino acid sequence consisting of amino acid residues 1 to 218 of SEQ ID NO:2.
6. An isolated nucleic acid molecule that is either (a) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:3, or (b) a nucleic acid molecule that remains hybridized following stringent wash conditions to a nucleic acid molecule consisting of the nucleotide sequence of nucleotides 225 to 821 of SEQ ID NO:1, or the complement of the nucleotide sequence of nucleotides 225 to 821 of SEQ ID NO:1.
7. The isolated nucleic acid molecule of claim 6, wherein any difference between the amino acid sequence encoded by the nucleic acid molecule and the corresponding amino acid sequence of SEQ ID NO:2 is due to a conservative amino acid substitution.
8. The isolated nucleic acid molecule of claim 6, comprising the nucleotide sequence of nucleotides 225 to 821 of SEQ ID NO:1.

9. A vector, comprising the isolated nucleic acid molecule of claim 8.
10. An expression vector, comprising the isolated nucleic acid molecule of claim 8, a transcription promoter, and a transcription terminator, wherein the promoter is operably linked with the nucleic acid molecule, and wherein the nucleic acid molecule is operably linked with the transcription terminator.
11. A recombinant host cell comprising the expression vector of claim 10, wherein the host cell is selected from the group consisting of bacterium, yeast cell, avian cell, fungal cell, insect cell, mammalian cell, and plant cell.
12. A method of producing Zsmf13 protein, the method comprising culturing recombinant host cells that comprise the expression vector of claim 10, and that produce the Zsmf13 protein.
13. The method of claim 12, further comprising isolating the Zsmf13 protein from the cultured recombinant host cells.
14. An antibody or antibody fragment that specifically binds with the polypeptide of claim 4.
15. A method of detecting the presence of *Zsmf13* RNA in a biological sample, comprising the steps of :
  - (a) contacting a *Zsmf13* nucleic acid probe under hybridizing conditions with either (i) test RNA molecules isolated from the biological sample, or (ii) nucleic acid molecules synthesized from the isolated RNA molecules, wherein the probe has a nucleotide sequence comprising a portion of the nucleotide sequence of the nucleic acid molecule of claim 8, or the complement of the nucleotide sequence of the nucleic acid molecule of claim 8, and
  - (b) detecting the formation of hybrids of the nucleic acid probe and either the test RNA molecules or the synthesized nucleic acid molecules,  
wherein the presence of the hybrids indicates the presence of *Zsmf13* RNA in the biological sample.
16. A method of detecting the presence of Zsmf13 in a biological sample, comprising the steps of:

- (a) contacting the biological sample with an antibody, or an antibody fragment, of claim 14, wherein the contacting is performed under conditions that allow the binding of the antibody or antibody fragment to the biological sample, and
- (b) detecting any of the bound antibody or bound antibody fragment.

17. A variant Zsmf13 polypeptide, wherein the amino acid sequence of the variant polypeptide shares an identity with the amino acid sequence of SEQ ID NO:2 selected from the group consisting of at least 70% identity, at least 80% identity, at least 90% identity, at least 95% identity, or greater than 95% identity, and wherein any difference between the amino acid sequence of the variant polypeptide and the amino acid sequence of SEQ ID NO:2 is due to one or more conservative amino acid substitutions.

18. A fusion protein, comprising the polypeptide of claim 4.

19. The fusion protein of claim 18, further comprising an immunoglobulin moiety.

## SEQUENCE LISTING

<110> ZymoGenetics, Inc.

<120> Human Semaphorin

<130> 99-69PC

<160> 5

<170> FastSEQ for Windows Version 3.0

<210> 1

<211> 821

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (168)...(821)

<221> misc\_feature

<222> (1)...(821)

<223> n = A,T,C or G

<400> 1

cggcggccgc	cccattcca	gaccggccgc	cagcccatct	ggtagctcc	cgccgctccg	60
cgccgcccgg	gagtcgggag	ccgcggggaa	ccgggcacct	gcaccgcct	ctggggggtc	120
ttctcccctg	tctgcctccc	ggagctagga	ctgcagaggg	gcctatc	atg gtg ctt	176
					Met Val Leu	

1

gca ggc ccc	ctg gct gtc	tcg ctg ttg	ctg ccc agc	ctc aca ctg	ctg	224
Ala Gly Pro	Leu Ala Val	Ser Leu Leu	Leu Pro Ser	Leu Thr	Leu Leu	
5	10	15				



2

gtg tcc cac ctc tcc agc tcc cag gat gtc tcc agt gag ccc agc agt 272  
 Val Ser His Leu Ser Ser Ser Gln Asp Val Ser Ser Glu Pro Ser Ser  
 20 25 30 35

gag cag cag ctg tgc gcc ctt agc aag cac ccc acc gtg gcc ttt gaa 320  
 Glu Gln Gln Leu Cys Ala Leu Ser Lys His Pro Thr Val Ala Phe Glu  
 40 45 50

gac ctg cag ccg tgg gtc tct aac ttc acc tac cct gga gcc cgg gat 368  
 Asp Leu Gln Pro Trp Val Ser Asn Phe Thr Tyr Pro Gly Ala Arg Asp  
 55 60 65

ttc tcc cag ctt gct ttg gac ccc tcc ggg aac cag ctc atc gtg gga 416  
 Phe Ser Gln Leu Ala Leu Asp Pro Ser Gly Asn Gln Leu Ile Val Gly  
 70 75 80

gcc agg aac tac ctc ttc aga ctc agc ctt gcc aat gtc tcn ctt ctt 464  
 Ala Arg Asn Tyr Leu Phe Arg Leu Ser Leu Ala Asn Val Xaa Leu Leu  
 85 90 95

cag gca aca gag tgg gcc tcc agt gag gac acg cgc cgc tcc tgc caa 512  
 Gln Ala Thr Glu Trp Ala Ser Ser Glu Asp Thr Arg Arg Ser Cys Gln  
 100 105 110 115

agc aaa ggg aag act gag gag gag tgt cag aac tac gtg cga gtc ctg 560  
 Ser Lys Gly Lys Thr Glu Glu Glu Cys Gln Asn Tyr Val Arg Val Leu  
 120 125 130

atc gtc gcc ggc cgg aag gtg ttc atg tgt gga acc aat gcc ttt tcc 608  
 Ile Val Ala Gly Arg Lys Val Phe Met Cys Gly Thr Asn Ala Phe Ser  
 135 140 145

ccc atg tgc acc agc aga cag gtg ggg aac ctc agc cgg act att gag 656  
 Pro Met Cys Thr Ser Arg Gln Val Gly Asn Leu Ser Arg Thr Ile Glu  
 150 155 160

aag atc aat ggt gtg gcc cgc tgc ccc tat gac cca cgc cac aac tcc 704  
 Lys Ile Asn Gly Val Ala Arg Cys Pro Tyr Asp Pro Arg His Asn Ser  
 165 170 175

aca gct gtc atc tcc tcc cag ggg gag ctc tat gca gcc acg gtc atc 752  
 Thr Ala Val Ile Ser Ser Gln Gly Glu Leu Tyr Ala Ala Thr Val Ile  
 180 185 190 195

gac ttc tca ggt cgg gac cct gcc atc tac cgc agc ctg ggc agt ggg 800  
 Asp Phe Ser Gly Arg Asp Pro Ala Ile Tyr Arg Ser Leu Gly Ser Gly  
 200 205 210

cca ccg cct cga gcc gaa ttc 821  
 Pro Pro Pro Arg Ala Glu Phe  
 215

<210> 2

<211> 218

<212> PRT

<213> Homo sapiens

<400> 2

Met Val Leu Ala Gly Pro Leu Ala Val Ser Leu Leu Leu Pro Ser Leu  
 1 5 10 15  
 Thr Leu Leu Val Ser His Leu Ser Ser Ser Gln Asp Val Ser Ser Glu  
 20 25 30  
 Pro Ser Ser Glu Gln Gln Leu Cys Ala Leu Ser Lys His Pro Thr Val  
 35 40 45  
 Ala Phe Glu Asp Leu Gln Pro Trp Val Ser Asn Phe Thr Tyr Pro Gly  
 50 55 60  
 Ala Arg Asp Phe Ser Gln Leu Ala Leu Asp Pro Ser Gly Asn Gln Leu  
 65 70 75 80  
 Ile Val Gly Ala Arg Asn Tyr Leu Phe Arg Leu Ser Leu Ala Asn Val  
 85 90 95  
 Ser Leu Leu Gln Ala Thr Glu Trp Ala Ser Ser Glu Asp Thr Arg Arg  
 100 105 110

Ser Cys Gln Ser Lys Gly Lys Thr Glu Glu Glu Cys Gln Asn Tyr Val  
 115 120 125  
 Arg Val Leu Ile Val Ala Gly Arg Lys Val Phe Met Cys Gly Thr Asn  
 130 135 140  
 Ala Phe Ser Pro Met Cys Thr Ser Arg Gln Val Gly Asn Leu Ser Arg  
 145 150 155 160  
 Thr Ile Glu Lys Ile Asn Gly Val Ala Arg Cys Pro Tyr Asp Pro Arg  
 165 170 175  
 His Asn Ser Thr Ala Val Ile Ser Ser Gln Gly Glu Leu Tyr Ala Ala  
 180 185 190  
 Thr Val Ile Asp Phe Ser Gly Arg Asp Pro Ala Ile Tyr Arg Ser Leu  
 195 200 205  
 Gly Ser Gly Pro Pro Pro Arg Ala Glu Phe  
 210 215

<210> 3

<211> 654

<212> DNA

<213> Artificial Sequence

<220>

<223> This degenerate sequence encodes the amino acid sequence of SEQ ID NO:2.

<221> variation

<222> (1)...(654)

<223> N is any nucleotide.

<221> misc\_feature

<222> (1)...(654)

<223> n = A,T,C or G

<400> 3

atggtnytng cnggnccnyt ngcngtnwsn ytnytntnc cnwsnytnac nytnytngtn	60
wsncayytnw snwsnwsnca rgaygtwnsn wsngarccnw snwsngarca rcarytntgy	120
gcnytnwsna arcayccnac ngtngcntty gargayytn arcctgggt nwsnaaytty	180

acntayccng gngcnmgnga yttywsncar ytngcnytn ayccnwsngg naaycarytn 240  
athgtngng cnmgnaayta yynttymgn ytnwsnytn cnaaygtnws nytnytncar 300  
gcnacngart gggcnwsnws ngargayacn mgnmgnwsnt gycarwsnaa rggnaaracn 360  
gargargart gycaraayta ygtnmngntn ytnathgtng cnggnmgnaa rgtnttyatg 420  
tgyggnaacna aygcnttyws nccnatgtgy acnwsnmgnc argtnggnaa yytnwsnmgn 480  
acnathgara arathaaygg ngtnngcnmgn tgyccntayg ayccnmgnca yaaywsnacn 540  
gcngtnathw snwsncargg ngarytntay gcngcnacng tnathgaytt ywsnggnmgn 600  
gayccngcna thtaymgnws nytnggnwsn ggncncnc cnmgngcnga rtty 654

&lt;210&gt; 4

&lt;211&gt; 15

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Peptide linker

&lt;400&gt; 4

Gly Gly Ser Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly  
1 5 10 15

&lt;210&gt; 5

&lt;211&gt; 1093

&lt;212&gt; PRT

&lt;213&gt; Mouse

&lt;400&gt; 5

Met Val Val Pro Gly Pro Leu Ala Leu Ser Leu Leu Leu Ser Ser Leu  
1 5 10 15  
Thr Leu Leu Val Ser His Leu Ser Ser Ser Gln Asp Ile Ala Ser Glu  
20 25 30  
Ser Ser Ser Glu Gln Gln Met Cys Thr Arg Arg Glu His Pro Ile Val  
35 40 45  
Ala Phe Glu Asp Leu Lys Pro Trp Val Phe Asn Phe Thr Tyr Pro Gly  
50 55 60



Ala Ala Trp Leu Pro Ile Ala Asn Pro Ile Pro Asn Phe Gln Cys Gly  
 355 360 365  
 Thr Leu Pro Glu Thr Gly Pro Asn Glu Asn Leu Thr Glu Arg Ser Leu  
 370 375 380  
 Gln Asp Ala Gln Arg Leu Phe Leu Met Ser Glu Ala Val Gln Pro Val  
 385 390 395 400  
 Thr Pro Glu Pro Cys Val Thr Gln Asp Ser Val Arg Phe Ser His Leu  
 405 410 415  
 Val Val Asp Leu Val Gln Ala Lys Asp Thr Leu Tyr His Val Leu Tyr  
 420 425 430  
 Ile Gly Thr Glu Ser Gly Thr Ile Leu Lys Ala Leu Ser Thr Ala Ser  
 435 440 445  
 Arg Ser Leu Arg Gly Cys Tyr Leu Glu Glu Leu His Val Leu Pro Pro  
 450 455 460  
 Gly Arg Leu Glu Pro Leu Arg Ser Leu Arg Ile Leu His Ser Ala Arg  
 465 470 475 480  
 Ala Leu Phe Val Gly Leu Ser Asp Arg Val Leu Arg Ile Pro Leu Glu  
 485 490 495  
 Arg Cys Ser Ala Tyr His Ser Gln Gly Ala Cys Leu Gly Ala Arg Asp  
 500 505 510  
 Pro Tyr Cys Gly Trp Asp Gly Lys Arg Gln Leu Cys Ser Thr Leu Glu  
 515 520 525  
 Asp Ser Ser Asn Met Ser Leu Trp Ile Gln Asn Ile Thr Thr Cys Pro  
 530 535 540  
 Val Arg Asn Val Thr Arg Asp Gly Gly Phe Gly Pro Trp Ser Pro Trp  
 545 550 555 560  
 Lys Pro Cys Glu His Leu Asp Gly Asp Asn Ser Gly Ser Cys Leu Cys  
 565 570 575  
 Arg Ala Arg Ser Cys Asp Ser Pro Arg Pro Arg Cys Gly Gly Leu Glu  
 580 585 590  
 Cys Leu Gly Pro Ser Ile His Ile Ala Asn Cys Ser Arg Asn Gly Ala  
 595 600 605  
 Trp Thr Ala Trp Ser Ser Trp Ala Gln Cys Ser Thr Ser Cys Gly Ile  
 610 615 620  
 Gly Phe Gln Val Arg Gln Arg Ser Cys Ser Asn Pro Ala Pro Arg His  
 625 630 635 640

Gly Gly Arg Ile Cys Val Gly Lys Ser Arg Glu Glu Arg Phe Cys Asn  
                                 645                                650                                655  
 Glu Asn Thr Pro Cys Pro Val Pro Ile Phe Trp Ala Ser Trp Gly Ser  
                                 660                                665                                670  
 Trp Ser Lys Cys Ser Asn Asn Cys Gly Gly Gly Val Gln Ser Arg Arg  
                                 675                                680                                685  
 Arg Ser Cys Glu Asn Gly Asn Ser Cys Pro Gly Cys Gly Val Glu Phe  
                                 690                                695                                700  
 Lys Thr Cys Asn Pro Glu Ala Cys Pro Glu Val Arg Arg Asn Thr Pro  
 705                                710                                715                                720  
 Trp Thr Pro Trp Leu Pro Val Asn Val Thr Gln Gly Gly Ala Arg Gln  
                                 725                                730                                735  
 Glu Gln Arg Phe Arg Phe Thr Cys Arg Ala Pro Leu Pro Asp Pro His  
                                 740                                745                                750  
 Gly Leu Gln Phe Gly Lys Arg Arg Thr Glu Thr Arg Thr Cys Pro Ala  
                                 755                                760                                765  
 Asp Gly Thr Gly Ala Cys Asp Thr Asp Ala Leu Val Glu Asp Leu Leu  
                                 770                                775                                780  
 Arg Ser Gly Ser Thr Ser Pro His Thr Leu Asn Gly Gly Trp Ala Thr  
 785                                790                                795                                800  
 Trp Gly Pro Trp Ser Ser Cys Ser Arg Asp Cys Glu Leu Gly Phe Arg  
                                 805                                810                                815  
 Val Arg Lys Arg Thr Cys Thr Asn Pro Glu Pro Arg Asn Gly Gly Leu  
                                 820                                825                                830  
 Pro Cys Val Gly Asp Ala Ala Glu Tyr Gln Asp Cys Asn Pro Gln Ala  
                                 835                                840                                845  
 Cys Pro Val Arg Gly Ala Trp Ser Cys Trp Thr Ala Trp Ser Gln Cys  
                                 850                                855                                860  
 Ser Ala Ser Cys Gly Gly Gly His Tyr Gln Arg Thr Arg Ser Cys Thr  
 865                                870                                875                                880  
 Ser Pro Ala Pro Ser Pro Gly Glu Asp Ile Cys Leu Gly Leu His Thr  
                                 885                                890                                895  
 Glu Glu Ala Leu Cys Ser Thr Gln Ala Cys Pro Glu Gly Trp Ser Leu  
                                 900                                905                                910  
 Trp Ser Glu Trp Gly Val Cys Thr Glu Asp Gly Ala Gln Ser Arg Ser  
                                 915                                920                                925

Arg Ser Cys Glu Glu Leu Leu Pro Gly Pro Gly Ala Cys Val Gly Asn  
930 935 940  
Ser Ser Gln Ser Arg Pro Cys Pro Tyr Ser Glu Ile Pro Val Ile Leu  
945 950 955 960  
Pro Ala Ser Ser Val Glu Glu Thr Thr Ser Cys Gly Gly Phe Asn Leu  
965 970 975  
Ile His Leu Ile Val Thr Gly Val Ser Cys Phe Leu Val Ser Gly Leu  
980 985 990  
Leu Thr Leu Ala Val Tyr Leu Ser Cys Gln His Cys Gln Arg Gln Ser  
995 1000 1005  
Gln Glu Ser Thr Leu Val His Pro Ala Thr Pro Asn His Leu His Tyr  
1010 1015 1020  
Lys Gly Gly Gly Thr Pro Lys Asn Glu Lys Tyr Thr Pro Met Glu Phe  
1025 1030 1035 1040  
Lys Thr Leu Asn Lys Asn Asn Leu Ile Pro Asp Asp Arg Ala Asn Phe  
1045 1050 1055  
Tyr Pro Leu Gln Gln Thr Asn Val Tyr Thr Thr Thr Tyr Tyr Pro Ser  
1060 1065 1070  
Pro Leu Asn Lys Pro Ser Phe Arg Pro Glu Ala Ser Pro Gly Gln Arg  
1075 1080 1085  
Cys Phe Pro Asn Ser  
1090



## INTERNATIONAL SEARCH REPORT

 Interna      Application No  
 PCT/US 00/30061

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 7    C07K14/47    C12N15/12    C12N15/63    C07K16/18    C12Q1/68 C07K19/00    G01N33/53		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 7    C07K    C12N    C12Q    G01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, WPI Data, PAJ, BIOSIS		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ADAM R.H. ET AL.: "A novel class of murine semaphorins with homology to thrombospondin is differentially expressed during early embryogenesis" MECHANISMS OF DEVELOPMENT, vol. 57, 1996, pages 33-45, XP000926255 cited in the application page 44, column 2, paragraph 3; figure 1 <div style="text-align: center;">--- -/--</div>	6
<div style="display: flex; justify-content: space-between;"> <span><input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.</span> <span><input checked="" type="checkbox"/> Patent family members are listed in annex.</span> </div>		
* Special categories of cited documents : <div style="display: flex;"> <div style="flex: 1;">           *A* document defining the general state of the art which is not considered to be of particular relevance            *E* earlier document but published on or after the international filing date            *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)            *O* document referring to an oral disclosure, use, exhibition or other means            *P* document published prior to the international filing date but later than the priority date claimed         </div> <div style="flex: 1;">           *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention            *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone            *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.            *&amp;* document member of the same patent family         </div> </div>		
Date of the actual completion of the international search	Date of mailing of the international search report	
29 March 2001	11/04/2001	
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer  Schönwasser, D	

## INTERNATIONAL SEARCH REPORT

Intern Application No

PCT/US 00/30061

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	NAGASE T ET AL: "PREDICTION OF THE CODING SEQUENCES OF UNIDENTIFIED HUMAN GENES.17. THE COMPLETE SEQUENCES OF 100 NEW CDNA CLONES FROM BRAIN WHICH CODE FOR LARGE PROTEINS IN VITRO" DNA RESEARCH, vol. 7, 2000, pages 143-150, XP000943428 ISSN: 1340-2838 Fig. 1, column 1, clone no. 2; Table 1, column 1, gene no. 2 -----	1-3,6,7, 9-12,14
A	US 5 639 856 A (MATTHES DAVID ET AL) 17 June 1997 (1997-06-17) SEQ ID NO:58 -----	1-19
A	SPRIGGS MELANIE K: "Shared resources between the neural and immune systems: Semaphorins join the ranks" CURRENT OPINION IN IMMUNOLOGY, vol. 11, no. 4, August 1999 (1999-08), pages 387-391, XP002158431 ISSN: 0952-7915 cited in the application the whole document -----	1-19
A	WO 99 45114 A (ZYMOGENETICS INC) 10 September 1999 (1999-09-10) the whole document -----	1-19

## INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No

PCT/US 00/30061

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5639856 A	17-06-1997	AU 683494 B	13-11-1997
		AU 7724094 A	03-04-1995
		CA 2171638 A	23-03-1995
		EP 0721342 A	17-07-1996
		JP 9505725 T	10-06-1997
		US 5935865 A	10-08-1999
		WO 9507706 A	23-03-1995
		US 6013781 A	11-01-2000
		US 5807826 A	15-09-1998
WO 9945114 A	10-09-1999	AU 2982799 A	20-09-1999

